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INNOCUOUS INTRACELLULAR ICE FORMATION: MECHANISMS AND IMPLICATIONS

by

JASON PAUL ACKER

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Successful cryopreservation of tissues has been limited by the lack of information on the mechanism by which these systems are damaged as a result of exposure to low temperatures. Intracellular ice formation (IIF), a potentially lethal form of cryoinjury, has been shown to occur more predominantly in biological systems with cell-cell and cell-surface interactions. Innocuous IIF has been proposed when the mechanism of ice formation does not result in permanent damage to the cell.

The work presented in this thesis represents a concerted effort to understand the mechanisms and implications of innocuous intracellular ice formation in tissue model systems. Investigations were conducted to explore the nature of the damage that results when ice forms within cells and the effect this intracellular ice has on the response of cells and tissue models to low temperatures. Fluorescent cryomicroscopy was utilized extensively to document the formation of intracellular ice in tissue models and the role of the plasma membrane in this process. By coupling cryomicroscopic studies with a progressive assessment technique, the effect of intracellular ice on cell viability was determined. The data collected from these experiments was combined into a conceptual model on the formation of intracellular ice in tissue models and the implications tested against a standard cryopreservation protocol.

The cell plasma membrane is a critical site of injury during the formation of intracellular ice. Innocuous IIF occurs when plasma membrane integrity is maintained by the propagation of intracellular ice between adjacent cells. This

process of intercellular ice propagation is facilitated by the presence of gap junctions. Innocuous intracellular ice affects the low temperature response of tissue models and protects the constituent cells from slow cooling injury by acting as an intracellular cryoprotectant. In the absence of chemical cryoprotectants, inducing intracellular ice formation is an effective method for the cryopreservation of tissue models.

With a better understanding of the protective mechanism of intracellular ice, development of techniques and procedures to minimize cryoinjury can be explored. These results provide researchers and clinicians new avenues to explore and new techniques to use in the development of the next generation of cryopreservation protocols.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

ATCC	American Type Culture Collection
CCD	charge-coupled device
EB	ethidium bromide
FDA	flourescein diacetate
IIF	intracellular ice formation
MDCK	madin darby canine kidney
Me_2SO	dimethyl sulfoxide
MEM	minimum essential media
PBS	phosphate buffered saline
TCM	tissue culture media
uv	ultraviolet

SYMBOLS

t_{50}	time for 50% intracellular ice formation
ΔT	difference in temperature
a	pore radius
L_f	molar latent heat of fusion
π^L	osmotic pressure of the solution
σ_{SL}	interfacial tension between the solid and liquid

T_f	freezing point of a planar ice-water interface
T_{fp}^{o}	freezing point of pure water
v_1	molar volume of pure water
v_2	molar volume of ice
v_3	molar volume of solution
θ	contact angle

CHAPTER ONE
INTRODUCTION

1.1 LIFE AND DEATH AT LOW TEMPERATURES

Since the first observation of "cellulae" by Sir Robert Hooke in 1665, scientists have endeavored to observe and document the complexity of living systems. Through careful experimentation and the development of precision instruments and staining techniques, detailed studies of the anatomy of the cell were made. In 1839, Schleiden and Schwann proposed that cells were the essential building blocks of all living material. It was thought that to understand the workings of a cell would be to understand the essence of life. However, it was not until scientists became interested in the manner in which cells and tissues function and interact with the environment that the concept of life and death would be explored.

As early as 1683, observations on the effects of cold suggested that exposure to low temperatures could both preserve life and cause death (11). It was observed that while some plants and animals could survive in freezing environments, most were lethally injured. It was thought that if one could identifying the factors which contribute to these organisms surviving freezing, then one would have discovered the elements responsible for life.

Motivated by the desire to understand the delicate relationship between freezing and death, a number of studies were performed to investigate the low temperature response of biological material. It was established that for most cells, death was not the result of simply cooling the sample below the freezing point of the solution (7,37). Using the precursor of the modern cryomicroscope, Göeppert in 1830 (21) and Molisch in 1897 (56) concluded that cell death was the result of the formation of ice. Subsequent studies focused on understanding the effects that ice formation had on cells and tissues.

In 1940, Luyet summarized the popular literature on the various theories related to the mechanism of injury and death by ice formation (40). In this review, he points out that the prevailing theories on the mechanism by which ice damages cells and tissues were based on two different types of ice formation. The first was the formation of extracellular ice that occurs during slow cooling, and the second was the formation of intracellular ice during rapid cooling. While in both cases lethal injury occurs to the cell, the proposed mechanisms by which injury results were different. During slow cooling, damage was the result of the excessive dehydration of the cell and the compression of the cells by extracellular ice (44,59). Intracellular ice results in cell death due to the mechanical disruption of the intracellular architecture (13,56,70). Luyet established that to understand the survival of cells and tissues following freezing and thawing one must consider the effects of these two mechanisms of ice formation.

Today, ice formation remains central to our understanding of the effects of low temperatures on the biological response of cells and tissues to freezing. However, unlike the early studies on the effects of ice formation on cell viability, we now appreciate that life can exist in the presence of ice. This must be qualified in that we now recognize that extracellular ice is not inherently lethal to cells and tissues. In fact, manipulating the degree of extracellular ice has been fundamental to the development of successful cryopreservation protocols. Intracellular ice formation, however, is not afforded the same respect.

The current tenet in the field of cryobiology is that intracellular ice is lethal. This belief has had an enormous influence on our understanding of the means by which cells and tissues are injured during freezing and on the development of techniques to prevent this damage. For the importance that this idea has had on cryobiology it is surprising that intracellular ice formation remains one of the most poorly understood physical phenomenon that occurs during freezing (16,26,57). Our understanding of intracellular ice has been limited to determining the conditions whereby it occurs so as to avoid those conditions during freezing.

Balancing the conditions where low temperatures can preserve life and cause death will require a complete understanding of the all of the elements involved in freezing. Expanding the information on intracellular ice formation will significantly contribute to the advancement of our understanding of life and death at low temperatures.

1.2 INTRACELLULAR ICE IS LETHAL – THE EVIDENCE

The conclusion that intracellular ice is lethal finds its origins in early work on the cold hardiness of plant tissue. Unraveling the resistance of plant tissue to low temperatures attracted many investigators to examine mechanisms of cryoinjury. Studies by the German researchers Göeppert (21), Müller-Thurgau (59,60) and Molish (56) observed that plant tissues were lethally injured when subjected to temperatures where congelation or intracellular freezing was observed. As subcooling, or supercooling, these plant tissues to low subzero temperatures did not result in significant damage, the presence of intracellular ice was identified as the injuring agent. These observations were quickly followed by a number of articles describing the formation of ice inside a variety of plant and animal cells (14,40,44,74). As the art of cryomicroscopy developed, more and more researchers began to correlate the presence of intracellular ice with lethal injury. From this work, numerous theories on the mechanism of damage began to appear in the literature (review 40).

In a seminal paper delivered in 1930, Stiles firmly established the lethal nature of intracellular ice (70). By this time, it was well recognized that intracellular ice formation would occur when cells or tissues were rapidly cooled below the freezing point of the extracellular solution. Validating this work, Stiles stated definitively that the formation of ice crystals in the protoplasm was responsible for the death of plants. As it was well established by this time that intracellular freezing was involved in cell death, the significance of this paper rests not in this statement, but in the proposed mechanism of damage the Stiles presented. He suggests that damage to plant cells was a result of the disruption of the internal architecture due to the formation of intracellular ice. Preventing this irreversible change in the structure of the cytoplasm by minimizing the formation of large ice crystals could protect the cell. Citing work by Tammann (71) on the relationship between cooling rate and ice crystal size, Stiles suggests that rapidly freezing a cell would minimize the amount of intracellular ice formed. In effect, Stiles was proposing that the formation of an amorphous state, or vitrification would protect cells from intracellular ice damage. This work by Stiles continues to be perpetuated in current theories on the effects of intracellular ice and in the attempts to avoid intracellular ice damage.

The assumption that intracellular ice was lethal and could be prevented through vitrification by rapid cooling was followed-up by the work of Luyet (34,35). In a series of papers Luyet described the effect of freezing conditions on the array if ice formed in biological systems (34,36,39,42). Using ultra-rapid freezing, early studies suggested that intracellular water could be vitrified as evident by the absence of ice crystals (43,55). This work prompted many individuals to investigate the use of rapid freezing for the preservation of biological material. Using controlled cooling rates in conjunction with the addition of protective chemicals, work with spermatozoa (38,61) established vitrification as a viable method for low temperature preservation. This early success using rapid cooling further entrenched the need to avoid intracellular freezing into current cryobiological thinking. Even today, vitrification remains one of the current approaches being developed for the cryopreservation of tissues and organs (17).

Interest in vitrification and intracellular ice faded following the discovery of the cryoprotectants glycerol (61) and dimethyl sulfoxide (32). Initial work with these cryoprotectants focus on the recovery of spermatozoa (38,61) and red blood cells (32). With the addition of cryoprotectants there was a marked improvement in the recovery of frozen biological material that were rapidly frozen and thawed. Surprisingly, cells that were rapidly frozen in the presence of glycerol did not contain intracellular ice, whereas the same cells frozen in the absence of glycerol did form intracellular ice (68). In 1953, Lovelock proposed that the recovery of red blood cells from rapid freezing in the presence of glycerol was not the result of vitrification, but the result of minimizing exposure to concentrated extracellular solutes (29,30,31). The cooling rate was introduced as an important factor in minimizing the effects of cryoprotectants and extracellular solutes (46). Increasing the cooling rate decreased the 'solution effects' injury. Due to the complexity involved in achieving vitrified solutions (53), vitrification was quickly substituted by studies on the effects of slow cooling and slow cooling injury.

In the late 1950's, Mazur began to develop what would later be termed the two-factor hypothesis of freezing injury (45,46,50). Prior work had identified that there was an optimal cooling rate for cell survival (41,54). Mazur proposed that this optimal cooling rate was a reflection of two different mechanisms of cell damage (45,46). Slow cooling increased the exposure of cells to concentrated solutes whereas rapid cooling resulted in intracellular ice formation. By examining the cooling rates that resulted in intracellular ice formation and the cooling rates the resulted in cell death, Mazur found a strong correlation between the two (47). He therefore concluded that the reduction in cell survival following rapid cooling was likely the result of intracellular ice.

From this early work, all efforts to examine the effects of intracellular ice indicated that it was lethal to cells and tissues. These results had a direct impact on the pioneering work that was being done at that time on the development of techniques for the cryopreservation of cells. All attempts were made to prevent the formation of intracellular ice by utilizing novel techniques for the addition and removal of cryoprotectants, and for the optimization of the cooling and warming rates. These slow cooling protocols are still in use today.

1.3 INNOCUOUS INTRACELLULAR ICE

The lethality of intracellular ice has been assumed to be an absolute. Any appearance of intracellular ice in a cell has been being equated with cell death. While the concept that intracellular ice is lethal has dominated past discussions on the role of rapid freezing, there has been an alternative perspective presented. In direct contrast, there are those that propose that the mere presence of intracellular ice is innocuous. These advocates of innocuous IIF suggest that amount of ice or size of the ice crystals (5,6,18,64,67,73), and the location of the ice formed (9,22,25,33,66) are more critical then the actual presence of intracellular ice. In this case, intracellular ice would not be an absolute determinant of lethal injury to a cell.

Innocuous intracellular ice formation has been demonstrated in cells and tissues where the amount of ice formed is minimized using rapid cooling (5,6,64,67). Minimizing the size of the ice crystals formed during ultra-rapid cooling and warming to the point that the sample remains transparent, has been shown to be successful at preserving tumor cell function (5,6,64). It is thought that there is a critical size of ice crystals that the cell can tolerate before damage to internal organelles occurs. Shimada has suggested that this critical size is 0.05 µm (67). Innocuous intracellular ice is therefore a reflection of the ability to maintaining the ice crystals below this critical size.

Historical attempts to obtain vitrified tissues using rapid freezing provide an excellent example of the success that researchers have had in minimizing the size of intracellular ice crystals and enhancing post-thaw survival (12,28,38). While the initial assumption was that ice formation had been prevented by rapid freezing (12,28) it was subsequently determined using x-ray diffraction that these cells did indeed form intracellular ice but that the crystal were too small to be resolved using microscopic techniques (43,47). Even today, there are frequent reports of tissues being vitrified using rapid freezing techniques with superior survival rates (15,76). As complete vitrification of bulk systems using traditional freezing protocols is unlikely (17,47,53), these results may be the product of "partial vitrification", or more correctly the presence of innocuous intracellular ice.

There have been a number of reported instances were innocuous intracellular ice formation has been demonstrated in cells and tissues where the ice crystals were much larger than the suggested critical size of 0.05 µm (22,33,65,66,72,73). In these cases, the amount of ice or the size of the intracellular ice crystals formed might not be as important as the location of ice within the cell. Studies have shown that the nucleus of cells is a targeted site for the formation of intracellular ice (1,9,33). Disruption of the sensitive structures found inside the nuclear membrane would seriously affect the ability of a cell to function. Studies have also shown that the membrane and membrane bound organelles are highly susceptible to damage from the formation of intracellular ice (20,33,65,66).

Localization of intracellular ice to non-critical sites may permit intracellular ice to be tolerated.

One of the most noted demonstrations of the innocuous nature of intracellular ice was performed by Mazur in 1960 (46). During the process of documenting the effects of freezing on the survival of yeast, Mazur observed that rapidly cooled cells could be 'rescued' by rapid warming. As the yeast cells formed intracellular ice on rapid cooling, the recovery of cells following rapid warming indicated that these cells were not damaged by the initial formation of intracellular ice. By correlating these results with observations made on the size of intracellular ice during freezing and thawing, Mazur concluded that cell damage was the result of recrystallization (47). While the exact mechanism by which recrystallization lethally injures cells has not be resolved, it has been proposed that damage may result from the physical rupture of intracellular organelles (47). Mazur's observations and conclusions exemplify the conflicting evidence that exists on the presence and mechanism of damage of intracellular ice.

There is considerable evidence to support the idea that the nucleation of intracellular ice is innocuous. As the mere presence of intracellular ice is not lethal, then protecting cells from rapid cooling injury involves more then just preventing the formation of intracellular ice. Understand the mechanism(s) by which damage occurs is the critical first step to resolving the ambiguities that exist regarding IIF.

1.4 INTRACELLULAR ICE FORMATION

The problem with the assumption that intracellular ice is lethal is that it is based solely on a series of causal relationships. It has been shown that intracellular ice occurs during rapid freezing (13,21,37,56) and that rapid freezing causes cell death (40,47). It has therefore been assumed that intracellular ice causes cell death (16,26,47,48,49,52,57). However, there is no direct evidence in the literature to lead one to conclude that the presence of ice within a cell is lethal. In fact, observations of innocuous intracellular ice provide direct evidence against this assumption. By focusing on the intracellular ice, researchers have neglected to consider the biological response of the cell to the significant environment changes that occur during the formation of intracellular ice. Cell death may not be a result of the intracellular ice, but a failure of the cell to respond favorably to the conditions surrounding intracellular ice formation. This is reflected in the statement by Mazur:

> *"...to state that the killing of rapidly cooled cells is due to intracellular ice is not the same as stating that intracellular ice formation is invariably lethal... the demonstration that when death occurs it is due to intracellular ice says nothing about the mechanisms by which intracellular ice injuries.*" (47)

One of the outstanding problems in cryobiology has been the difficulty in differentiating between the presence of intracellular ice and the process of intracellular ice formation. Developing techniques that can separate the results of one from the other have so far eluded researchers. This is clearly evident in the fact that we do not know if intracellular ice formation is the cause of (16,19,48) or results from (58,69) damage to the cell. This has significantly limited our ability to effectively utilize rapid freezing protocols for the cryopreservation of cells and tissues.

1.5 INTRACELLULAR ICE FORMATION IN TISSUES

The unique physical properties of tissues have been shown to significantly effect the biological response to freezing. The diversity of cell types and cell densities as well as the morphological differences between constituent cells significantly effects the osmotic and thermal state of a tissue (27). This has dramatic implications on the cooling rates that can be attained and hence the response of the tissue to freezing and thawing. In addition, the requisite cell-cell and cell-matrix interactions in a tissue have been implicated in the poor survival of tissues following freezing (2,3,4,8,24,75). The presence of cell junctions has been shown to significantly reduce the optimal cooling rate for the preservation of tissue function (4,10,23,24). It has been proposed that this affect on cooling rate is the result of an increased incidence of intracellular ice formation (4,10,23,24). IIF has been shown to be significantly effected by the presence and type of cellular junctions (1,8,62). From the literature, one can concluded that the freezing response of tissues, and the mechanism by which damage occurs are affected by the physical structures unique to tissues.

The governing principle in the cryopreservation of tissues is that intracellular ice formation is lethal. During freezing, efforts are taken to either cool very rapidly and avoid ice formation (vitrification), or to cool slowly and minimize the amount of ice formed intracellularly (51). Due in part to the highly successful application of the above two ice-limiting techniques in cellular systems, the idea of innocuous ice formation has been relatively ignored. In tissues, where there is a significant degree of interstitial and intracellular ice formation as a result of the tissue structure and the inhibited heat and mass transfer, minimization of extra- and intracellular ice in specific locations of the cells and tissues may be more important than complete elimination (27). A detailed examination of intracellular ice formation in tissues is therefore warranted to determine the nature of damage caused by the presence of ice, to examine limits to the amounts of tolerable ice, and to develop strategies for cryopreservation.

Evidence in the literature shows that conditions exist where intracellular ice is not lethal. It is of interest to note that most of the documented cases involving the innocuous formation of intracellular ice have been in systems composed of cell aggregates or intact tissues (6,22,33,64,65,72). The discordance of opinions on lethal and innocuous IIF may be more to do with the differences between cell and tissue cryobiology than the intracellular ice itself. By understanding the mechanism by which intracellular ice forms, and the means by which it can remain innocuous, the potential exists for the development of novel approaches for the cryopreservation of tissues. However, mainstream acceptance of the concept of innocuous intracellular ice formation needs to occur first.

1.6 HYPOTHESIS

It is proposed that the damage to cells during rapid freezing is a result of the mechanism of intracellular ice formation and not the presence of intracellular ice crystals. The unique conditions whereby intracellular ice formation occurs in tissues will, therefore, not result in lethal injury to the constituent cells.

1.7 OBJECTIVES AND APPROACH

The successful cryopreservation of tissues has been limited by the lack of information on the mechanism by which these systems are damaged as a result of exposure to low temperatures. The formation of intracellular ice, a potentially lethal form of cryoinjury, has been shown to occur more predominantly in biological systems that possess cell-cell and cell-surface interactions (1). As the mechanism of intracellular ice formation dictates the degree of cell damage, the unique conditions whereby IIF occurs in tissue models systems may prevent injury. This thesis will explore the mechanisms and implications of innocuous intracellular ice formation in tissue model systems.

In order to prove the hypothesis, it will be necessary to show that a direct correlation exists between the mechanism of intracellular ice formation and lethal cell injury. By establishing this relationship it will then be possible to investigate the mechanism by which the formation of intracellular ice is affected by the unique properties of tissue model systems.

The objectives of this thesis are:

1. **To establish the innocuous nature of intracellular ice.**

   There is evidence to suggest that the formation of intracellular ice in tissue systems is innocuous (6,22,33,63,64,65,66). By coupling cryomicroscopy procedures with established viability assessment techniques we will correlate the presence of intracellular ice with post-thaw survival. Employing multiple assays that measure the activity or functionality of specific cellular components will allow us to identify the sites of freezing injury.

Cooling rate has been identified as an important factor in the low temperature response of cells (47). Damage to cells has been directly attributed to the rate of cooling (50) with an optimal rate being determined by the permeability properties of the cell (48,50,51). The presence of intracellular ice prior to cooling would significantly effect the efflux of water from the cell during cooling, and hence the conditions for cell damage. Using a graded freezing protocol, we will examine the effect that intracellular ice has on the biological response of tissue model systems to freezing.

2. **To develop an understanding of the mechanism(s) by which innocuous intracellular ice occurs in tissue model systems.**

Understanding the sequence of events surrounding the formation of intracellular ice would significantly enhance our understanding of the response of cells and tissues to rapid freezing. Critical to the mechanism of intracellular ice formation is the role of the cell plasma membrane (16,26,57). Using fluorescent cryomicroscopy we will examine the relationship between intracellular ice formation and membrane damage. Video analysis will be used to improve our knowledge of the spatial and temporal sequence of events involved in membrane damage and intracellular ice formation.

Cell-cell propagation of intracellular ice has been shown to significantly increase the susceptibility of confluent monolayers (1) and tissues (8) to IIF. While gap junctions have been implicated in the induction of intracellular ice between adjacent cells (1,8) the mechanism of ice induction has not been well established. We will combine theoretical and experimental evidence to demonstrate that gap junctions do have a role in the propagation of intracellular ice in tissues and tissue models.

The ability to predict the probability of IIF for a given freezing protocol has met with limited success because of an inadequate understanding of the mechanism(s) by which intracellular ice damages cells. We will summarize our current understanding of intracellular ice formation in tissue systems into a conceptual model. This will include information on the role of the cell plasma membrane, the effect of cell-cell propagation and the freezing response of tissue models. Experimental evidence will be used to validate the assumptions and verify the conclusions.

3. **To demonstrate the utility of intracellular ice formation in the cryopreservation of tissues.**

Current protocols for the cryopreservation of cells and tissues preclude the use of rapid cooling rates due to the lethal damage resulting from intracellular ice formation. Establishing the presence and mechanisms of formation of innocuous intracellular ice in tissue models implores the search for novel cryopreservation techniques. Comparisons with standard cryopreservation protocols will establish the utility of employing innocuous intracellular ice formation in the cryopreservation of tissues.

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CHAPTER TWO

INNOCUOUS INTRACELLULAR ICE FORMATION IN CONFLUENT CELL MONOLAYERS

2.1 INTRODUCTION

Critical to the development of effective cryopreservation strategies for cells and tissues is the elucidation of the mechanisms and conditions responsible for cell injury. Biophysical studies have provided a detailed understanding of the biological freezing response. Our understanding of cell damage during freezing is based on the two factor hypothesis of cryoinjury (38). During slow cooling, the cell is dehydrated as the extracellular solutes concentrate due to the removal of pure water from the solution in the form of ice. It has been proposed that damage results from the excessive dehydration of the cell and the increased intracellular and extracellular solute concentrations (29,38,43). During rapid cooling, the formation of extracellular ice and the concentration of solutes occurs too quickly for the cell to respond by exosmosis. This results in the cytoplasm becoming increasingly supercooled with a concomitant increase in the probability of freezing. This is termed intracellular ice formation (IIF). In cell suspensions, there is strong evidence to suggest that the formation and presence of intracellular ice is lethal (16,26,32,37). However, the mechanism by which IIF damages cells has not been conclusively determined (16,26,45). Regardless, cryopreservation protocols have focused on optimizing the cooling rate to minimize slow cooling injury while avoiding the formation of intracellular ice.

One of the confounding problems in cryobiology is the phenomenon of intracellular ice formation. There are those that conclude that intracellular ice formation is lethal and should be avoided during freezing (13,32,35,37,59) and there are those who have determined that intracellular ice is innocuous (9,30,49,54,55,56,61,62). To unravel this seemingly contradictory issue cryobiologists need to determine whether IIF is the cause of injury to the cell or a result of damage that precedes the formation of intracellular ice. To date, researchers have been unable to solve this problem. As a result of the lack of clarity on this issue, the worst case scenario has been adopted – that intracellular ice is lethal.

The problem with the assumption that intracellular ice is lethal is that it is based solely on a series of causal relationships. It has been shown that intracellular ice formation occurs during rapid freezing (13,31,44) and that rapid freezing causes cell death (32,35). It has therefore been assumed that intracellular ice formation causes cell death (16,26,35,36,37,42,45). However, there is no direct evidence in the literature to lead one to conclude that the presence of ice within a cell is lethal. In fact, the observations of innocuous intracellular ice provide direct evidence against this assumption. So the question remains what is the relationship between intracellular ice and cell death?

The current understanding is that the plasma membrane is an integral element in the formation of intracellular ice and the subsequent injury to cells. For intracellular ice to form within a cell, two general conditions must be met: the cell cytoplasm must be supercooled, and a nucleating event must occur. Supercooling of the cytoplasm results during rapid cooling when the cell is unable to dehydrate sufficiently to maintain the chemical potential of intracellular water close to that of the unfrozen extracellular solution (28,35). The permeability of the plasma membrane to water and solutes is therefore critical in this process. The mechanism by which the supercooled cytoplasm becomes nucleated has not been resolved (16,26,45). As the plasma membrane is an effective barrier to the propagation of extracellular ice (13,31,34) and there are few efficient nucleators in a cell (51), it has been suggested that extracellular ice is involved in the nucleation of the cytoplasm (26,31,34). The interaction of extracellular ice and the cell plasma membrane is therefore critical to the formation of intracellular ice (16,26,34,45,46,60). Understanding this interaction will therefore be important in elucidating the mechanism of intracellular ice formation and damage that result.

The avoidance of intracellular ice formation has been a common practice in the cryopreservation of cell suspensions. However, recent studies with tissue model systems suggest that this may be much more difficult during tissue cryopreservation. It has been demonstrated in cultured hepatocytes (12,25), fibroblasts (1,4) and keratinocytes (7) that there is a significant increase in the formation of intracellular ice at lower cooling rates and at higher subzero temperatures than occurs in the same cells in suspensions. It has further been reported that cell-cell contact can facilitate the nucleation of ice between adjacent cells (1,3,10). The capacity for a cell to induce ice formation in an adjoining cell will enhance the incidence of intracellular ice in tissues. An understanding of the mechanism by which damage due to IIF occurs would facilitate the development of techniques for its minimization or avoidance during tissue cryopreservation.

Intracellular ice may not be lethal. The fact that rapidly cooled cells can survive if rapidly warmed suggests that the ice itself may not be damaging (33,35). The amount of ice (20,37,56,62), the size of the ice crystals (9,52,56), and the location of the ice formed (11,30,54,55) have been identified as conditions that contribute to cellular damage. There have been numerous studies suggesting that innocuous intracellular ice formation is possible when the amount of ice formed is controlled (56,62). Minimizing the size of the ice crystals formed during rapid cooling and warming by ensuring that the sample remains transparent, has been shown to be successful at preserving tumor cell function (9,52,56). However, the amount of ice and the size of the ice crystals formed might not be as important as the location within the cell. Studies have shown that the plasma membrane and membrane bound organelles are more susceptible to the formation of intracellular ice (30,54,55). Disruption of the sensitive structures found inside the nuclear membrane or disruption of the mitochodria would seriously affect the ability of a cell to function.

Evidence in the literature shows that conditions exist where intracellular ice is not lethal. It is of interest to note that most of the documented cases involving the innocuous formation of intracellular ice have been in systems composed of multiple cells or intact tissues (9,24,30,52,54,61). The discordance of opinions on lethal and innocuous IIF may have more to do with the differences between cell and tissue cryobiology than the intracellular ice itself. By understanding the mechanism by which intracellular ice forms, and the means by which it can remain innocuous, the potential exists for the development of novel approaches for the cryopreservation of tissues. However, mainstream acceptance of the concept of innocuous intracellular ice formation needs to occur first.

The purpose of this study is to examine the effect that the presence of intracellular ice has on the post-thaw survival of two different cell types cultured as two distinct experimental models (single attached cells and confluent monolayers).

2.2 MATERIALS AND METHODS

Cell Culture

Two cell lines were used to investigate the effect of intracellular ice formation on cell viability. The first was the Madin Darby Canine Kidney (MDCK; CCL34 ATCC) epithelial cell line. These cells were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential media (MEM) supplemented with 10% v/v fetal bovine serum (all components from GIBCO Laboratories, Grand Island, NY). Cells were grown in tissue culture flasks (25 cm^2; Corning Glass Works, Corning, NY) and harvested by exposure to a 0.25% trypsin-EDTA solution (GIBCO) for 10 min at 37°C. The MDCK cells were re-suspended in supplemented MEM to obtain cell suspensions and then plated on sterilized cover slips (12 mm circle, Fisher Brand) at a concentration of $2x10^4$ cells/mL. The cover slips were kept in petri dishes in an incubator for 12 h to allow the cells to attached and 3 d to allow the growth of a confluent monolayer.

The second cell line was the V-79W line of Chinese hamster fibroblasts. Cells were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential medium (MEM) with Hanks' salts, 16 mmol/L sodium bicarbonate, 2 mmol/L L-glutamine and 10% fetal bovine serum supplemented with antibiotics (penicillin G (50 units/mL), streptomycin (50 µg/mL)) (all components from GIBCO Laboratories, Grand Island, NY). Cells were grown in tissue culture flasks (25 cm^2; Corning Glass Works) and harvested by exposure to a 0.25% trypsin solution (GIBCO) for 10 min at 37°C. The fibroblasts were resuspended in supplemented MEM. Sterilized cover slips (12 mm circle, FISHER Brand) were placed in a petri dish (FISHER Brand, 100x15 mm) and covered with 15 mL of supplemented MEM containing $3x10^5$ cells. The petri dishes were incubated for 12 h to allow the cells to attach and 3 days to allow the growth of a confluent monolayer.

The V-79W and MDCK cell lines are established cell lines that have been studied extensively in cryobiology, with the osmotic and cryobiological responses having been previously determined (1,2,3,4,5,38,39,41,45,64). By carefully manipulating the culture conditions, these two cell lines can be used to model the distinct morphology and structure of simple tissues. Single attached cells are characterized by cell-surface adhesion but no cell-cell interactions. Binding to a substrate result in cells assuming a flat, non-spherical shape where one face is excluded from exchange with the extracellular medium. Confluent monolayers result when cells attach and divide forming both cell-surface and cell-cell interactions. Confluent monolayers will be used to examine the influence of cell-cell interactions on the formation of intracellular ice. It is recognized that cellular properties and responses may be different for exponentially growing single attached cells and the confluent monolayers.

When cultured to confluency, V-79W hamster fibroblasts will form cell-cell adhesions, but will not form gap junctions (1,2,5,64). MDCK epithelial cells that are cultured to confluency will form cell-cell adhesions, including gap junctions (1,2,5,64). This difference in the type of cell-cell adhesions expressed in V-79W and MDCK confluent monolayers will be used to investigate the effect that gap junctions have on the formation of intracellular ice.

Cryomicroscope and Video System

The cryomicroscope and video system used for this study is described in detail elsewhere (5,45). Briefly, it consisted of a Zeiss fluorescent microscope (Carl Zeiss, Germany), a CCD video camera (ZVS-47DEC, Carl Zeiss), a video recorder (GX4, Panasonic, Japan) and a convection cryostage similar to the design described by Diller *et al.* (15). The cryostage was connected to a computer-controlled interface (Great Canadian Computer Company, Spruce

Grove, Canada). The computer monitored the temperature by analyzing the voltage from a thermocouple on the stage and via a proportional controller circuit, heat was added as necessary to allow the stage to follow a user defined thermal protocol.

Progressive Assessment of Cell Viability

A progressive assessment technique was used to assess the post-thaw survival of V-79W and MDCK single attached cells and confluent monolayers. The progressive assessment technique is described in detail in Appendix A. This procedure combines the assessment of intracellular ice formation, membrane integrity, metabolic activity and clonogenic function into a single process that can be performed on an individual experimental sample. Any cell-type specific changes to the progressive assessment technique are outlined below.

Intracellular Ice Formation: The formation of intracellular ice was detected using the standard flash method (13,31) in conjunction with a fluorometric technique (5,30). The fluorescent nucleic acid probe SYTO 13 (Molecular Probes, Eugene, OR) was used to assist in the identification of IIF in the MDCK and V-79W confluent monolayers. The formation of intracellular ice was determined to occur if there was a distinct disruption in the pattern of fluorescent staining upon freezing. Single attached cells and confluent cell monolayers were stained with SYTO and allowed to incubate at 22°C for 10 min (MDCK) or 2 min (V-79W) prior to freezing. The incidence of IIF was calculated as the percent of cells that form intracellular ice compared to total cells in the sample.

Membrane Integrity: A dual fluorescent staining technique was used for the quantitative assessment of the integrity of the cell plasma membrane. SYTO 13, a permeant live cell nucleic acid dye (1.25 μM) and ethidium bromide (EB; Sigma Chemical Company, Mississuaga, ON; 2.5 μM) where used to differentially stain the cells. Percent survival based on membrane integrity was calculated as the number of SYTO positive cells over the total number of cells (SYTO and EB positive) using equation A-1.

Metabolic Activity: AlamarBlue™ (Biosource International, CA) was used to assess the overall metabolic activity of the confluent monolayers and single attached cells post-thaw. AlamarBlue is an oxidation-reduction indicator that is dependent on the maintenance of a functional electron transport pathway (48) and hence an intact and functioning mitochondria. AlamarBlue was added to tissue culture media (5% v/v) and confluent monolayers (MDCK and V-79W) were incubated in this solution for 12 hours at 37°C. Single attached cells (MDCK and V-79W) were incubated for 24 hours at 37°C in 5% alamarBlue. An aliquot (100 µL) of the media was removed and measured on a spectrophotometer (570-600 nm; UVmax, Molecular Dynamics, CA). Percent survival based on metabolic activity was calculated as the mean percent difference in reduction between the experimental samples and the controls using equation A-2 and A-3.

Clongenic Assessment: The cells were collected from the coverslips by exposure to a 0.25% trypsin-EDTA solution and diluted to 750 cells/mL. Tissue culture flasks were seeded with 150 cells/flask and incubated at 37°C for 5 days. The tissue culture media was removed and the colonies were fixed with 70% isopropanol, stained with trypan blue, and rinsed with distilled water before being counted. Percent survival based on clonogenic function was calculated as the mean of the experimental colony counts expressed as a percentage of the mean controls.

Overall Experimental Design

Madin-Darby canine kidney (MDCK) epithelial cells and V-79W hamster fibroblasts were either attached individually or grown to confluency on glass coverslips, then stained with SYTO and EB prior to freezing. The samples were then supercooled to a defined subzero experimental temperature on a convection cryostage by cooling at 25°C/min. The subzero experimental temperature was carefully chosen to ensure that approximately 100% of the cells would form intracellular ice (Figure 2.1). Under video surveillance, ice was nucleated using a cold copper probe at the constant temperature and the incidence of intracellular ice formation was observed after holding for 5 min. Cells were then warmed at 25°C/min and the integrity of the cell plasma was quantitatively assessed. The samples were then transferred from the cryomicroscope and assayed for metabolic function using the reduction-oxidation indicator alamarBlue. The cells were trypsinized, plated, incubated for 5 days, and the number of colony forming units was determined. The cumulative incidence of cells with intracellular ice formation was determined as a function of nucleation temperature and correlated with post-thaw survival.

Statistical Analysis

Throughout the study numerous statistical comparisons were performed. Unless otherwise indicated, a one way ANOVA was used with a level of significance set at 0.05.

2.3 RESULTS

Effect of Cell-Cell and Cell-Surface Adhesions on IIF

The incidence of intracellular ice in V-79W and MDCK cells is strongly dependent on the presence of cell-cell and cell-surface adhesions and the temperature of nucleation (Figure 2.1). In this study, nucleation temperatures were chosen to ensure that 100% of the cells would form intracellular ice regardless of cell type or morphological configuration.

Effects of Intracellular Ice Formation in Single Attached Cells

The incidence of intracellular ice formation, membrane integrity, metabolic activity and clonogenic function at different nucleation temperatures for V-79W and MDCK single attached cells is shown in figure 2.2 and 2.3 respectively. At the experimental temperatures chosen, approximately 100% of the cells formed intracellular ice in both of the cell lines studied. Immediately post-thaw, there was a significant reduction in the number of cells with intact cell plasma membranes. In V-79W, 5.3 ± 2.3% of the cells have an intact membrane following ice nucleation at –10°C. The membrane integrity of MDCK single attached cells ranges from 7.2 ± 7.2% at –8 °C to 0% at –12 °C. This data shows disruption of the plasma membrane following IIF in MDCk and V-79W single attached cells.

Reduction in metabolic activity assessed using alamarBlue followed a similar pattern. Incubation at 37°C in 5% alamarBlue revealed that the V-79W and MDCK single attached cells displayed a lower level of metabolic activity compared to unfrozen controls. The metabolic activity of V-79W cells was 2.8 ± 2.0% at –10°C and 0% at –12°C. MDCK cells had a metabolic activity ranging from 10.5 ± 3.1% at –8°C to 9.9 ± 3.7% at –12°C. The formation of intracellular ice significantly reduced the post-thaw metabolic activity of V-79W and MDCK single attached cells.

After the evaluation of metabolic activity, single attached cells were examined for clonogenic function using a standard colony-forming assay. Following trypsinization, plating and incubation for 5 days, both V-79W and MDCK cells displayed no clonogenic function at the nucleation temperatures used in this study.

Statistical analysis revealed that there was no difference between the survival based on membrane integrity, metabolic activity or clonogenic function at each experimental temperature for V-79W and MDCK single attached cells. Following intracellular ice formation there was a significant reduction in the three indicators of cell viability.

Monolayers and Intracellular Ice Formation

V-79W and MDCK confluent cell monolayers were subjected to low temperature conditions where intracellular ice formation was known to occur and then evaluated using a progressive assessment technique. The effect of IIF on the post-thaw survival of V-79W and MDCK confluent monolayers is shown in Figure 2.4 and 2.5 respectively. As MDCK monolayers have been shown to form intracellular ice at high subzero temperatures (1,2), it was possible to extend the range of nucleation temperatures used. At the experimental temperatures chosen, approximately 100% of the cells formed intracellular ice in both V-79W and MDCK monolayers.

The pattern of ice propagation through the MDCK monolayers was significantly different than that in the fibroblast monolayers. In the V-79W monolayer, the passage of the ice front through the field of view was later followed by IIF in a number of randomly dispersed cells. This initial formation of ice was proceeded by the induction of ice from one cell to another resulting in clusters of cells displaying IIF. This was identical to the report (3) that cell-cell contact can facilitate the nucleation of intracellular ice between adjacent fibroblast cells. In the MDCK monolayer, however, the passage of the ice front was quickly followed by IIF in all the cells in the monolayer in a wave-like manner in the same direction as the ice front. It has been proposed that by facilitating the nucleation of ice between adjoining cells, intercellular contact enhances the incidence of intracellular ice in confluent monolayers (1).

Post-thaw assessment of membrane integrity revealed that the majority of the constituent cells of both V-79W and MDCK monolayers had an intact cell plasma membrane (Figure 2.4 and 2.5). For V-79W monolayers, the incidence of an intact membrane was 79.5 ± 6.8% at –10°C and 81.5 ± 4.8% at –12°C. The incidence of membrane integrity ranged from 82.4 ± 4.2% at –5°C to 75.2 ± 4.7 at –11°C in MDCK confluent monolayers. There was no statistical difference in the incidence of membrane integrity across nucleation temperatures for V-79W and MDCK monolayers.

Metabolic activity of V-79W and MDCK monolayers was measured following the formation of intracellular ice. The metabolic activity of V-79W monolayers was 82.0 ± 8.6% at –10°C and 73.4 ± 11.1% at –12°C. MDCK cells had a metabolic activity ranging from 89.7 ± 5.2% at –5°C to 91.9 ± 5.0% at –11°C. The temperature of nucleation was not a significant factor in the post-thaw metabolic activity of V-79W and MDCK monolayers.

The clonogenic function of V-79W and MDCK confluent cell monolayers was only slightly affected by the formation of intracellular ice. V-79W monolayers displayed a clonogenic function of 75.8 ± 7.7% and 72.4 ± 8.0% following nucleation at -10°C and –12°C, respectively. Compared to unfrozen controls, the clonogenic function of MDCK monolayers ranged from 72.0 ± 13.9% at –5°C to 75.8 ± 12.8% at –11°C. There was no statistically significant difference in the clonogenic function with nucleation temperature for V-79W and MDCK monolayers.

Neither the formation nor presence of intracellular ice had a detrimental affect on the membrane integrity, metabolic activity or clonogenic function of V-79W and MDCK confluent monolayers. Approximately 80% of the cells from V-79W and MDCK monolayers were 'viable' following intracellular ice formation.

2.4 DISCUSSION

The purpose of this study was to investigate the effect of intracellular ice formation on cell viability. Using two different cell models, single attached cells and confluent monolayers, the effect of cell morphology and physiological condition on the incidence of intracellular ice formation could be assessed.

The progressive assessment technique allowed for the assessment of membrane integrity, metabolic activity and clonogenic function of single attached cells and confluent monolayers following intracellular ice formation. This tool permitted assessment of the same experimental sample using these three different techniques. By incorporating a number of different assays into a single experimental procedure, limitations and errors associated with one technique were offset by the use of the others (Appendix A).

Single Attached Cells are Lethally Injured by IIF

The progressive assessment of post-thaw membrane integrity, metabolic activity and clonogenic function following IIF showed significant reductions in these viability parameters in both V-79W and MDCK single attached cells. Acker and McGann (5) reported that, as the incidence of IIF increased in single attached cells, there was a parallel increase in the damage to the plasma membrane. This study supports the observation by Acker (1) that there is a strong correlation between the formation of intracellular ice and membrane damage in single attached cells.

Both V-9W and MDCK single attached cells demonstrate a decreased metabolic activity following intracellular ice formation indicating mitochondrial damage. As the alamarBlue assay tests is dependent on the maintenance of a functional electron transport pathway (48) and hence intact mitochondria, the absence of metabolic activity is a strong indicator of damage to this organelle. It has been previously reported that intracellular freezing can have detrimental effects on the structure and function of organelles (18,19,37), specifically the lysosomes and mitochondria (41). The observed decrease in metabolic activity of single attached cells supports the concept that intracellular ice formation may disrupt critical intracellular structures. However, the question remains whether it is the intracellular ice that damages these intracellular structures or the process of ice formation in the cell that is the damaging event in this experimental system.

The residual metabolic activity (<10%) in the V-79W and MDCK single attached cells following nucleation indicates either some intact mitochondria are still present or there are residual enzymes in the extracellular solution. There is evidence in the literature to suggest that both of these possibilities may be true. In microbial systems, cell numbers are significantly decreased following freezing, however, respiratory activity can be sustained and even enhanced (35,47,53). In addition, work with the catalase enzyme in yeast has demonstrated higher enzymatic activity in frozen-thawed cells compared to unfrozen cells (35,47). This supports the concept that enzymatic and respiratory activity can be maintained in cells that are damaged during freezing. However, in this study, it is equally likely that a small percentage of cells survived IIF and are metabolically active resulting in the observed reaction.

While membrane integrity and metabolic activity are strong indicators of cell recovery following freezing, the definitive test for cell viability is clonogenic function (35,39,41). The ability to interact with the environment through attachment to a substrate, and the capacity to grow and divide are only possible if the cell is physiologically and biochemically stable. V-79W and MDCK single attached cells with IIF showed no clonogenic function. As the post-thaw incubation period would permit the repair of any sublethal freeze-thaw damage

(40), the absence of clonogenic function indicates that IIF irreversibly damages critical cellular structures.

This data clearly demonstrates that IIF is lethal in V-79W and MDCK single attached cells.

Innocuous IIF in Confluent Cell Monolayers

Intracellular freezing does not result in significant damage to V-79W or MDCK confluent monolayers. Using the progressive assessment technique, it was shown that constituent cells of confluent monolayers could tolerate intracellular ice and the process of intracellular ice formation.

Intracellular ice formation does not result in the immediate disruption of the cell plasma membrane following warming of monolayers. Post-thaw, there was a high proportion of cells with an intact cell plasma membrane (>80%) in MDCK and V-79W monolayers. This is consistent with the report by Acker and McGann (5) who observed a similarly high recovery of membrane integrity after intracellular ice formation in V-79W monolayers. As plasma membrane integrity has been used as an initial assessment of cell viability (34,35,41,64), this data would suggest that the confluent monolayers are 'viable' following intracellular freezing. Similarly, there was a high recovery of metabolic activity after IIF in both V-79W and MDCK monolayers (Figure 2.4 and 2.5). This indicates the presence of intact mitochondria and a functioning electron transport chain following the formation or presence of ice in the cell.

Not only was the metabolic activity maintained following intracellular freezing, in most cases recovery of metabolic activity was somewhat higher than that for membrane integrity and clonogenic function. This heightened activity may be an indicator of an increase in the overall cell metabolism in response to freeze-thaw damage. Repair of sublethal injury following freezing (37,39,40) would be an example where an elevation in cellular energy production and metabolic activity would occur. However, the difference between membrane integrity and metabolic activity was not significant, suggesting that this increased response could equally be attributed to experimental error.

The final assessment of cell viability following intracellular freezing was to examine the reproductive integrity of the individual cells of the confluent monolayer. The results from the clonogenic functional test indicate that the majority (<70%) of the constituent cells of the V-79W and MDCK confluent monolayers are viable, and that there was no secondary injury resulting from the formation of intracellular ice. IIF does not confer lethal damage to all of the V-79W and MDCK cells in confluent monolayers.

The presence of intracellular ice in V-79W and MDCK confluent cell monolayers is innocuous.

Role of Plasma Membrane in Intracellular Ice Damage

The response of cells to freezing depends critically on the properties of the cell plasma membrane. As a barrier to ice propagation (13,34) and a primary area of contact between a cell and the environment, the plasma membrane modulates cellular responses to the physical and chemical changes that occur as ice forms extracellularly. Traditionally, plasma membrane integrity has been used as an initial indicator of cell viability following exposure to low temperatures (5,7,41,64). A damaged plasma membrane and subsequent loss of semipermeability are indicators of lethal injury to a cell (4,14,34,35,64).

One of the few consistent elements in the study of IIF has been the observation that following warming, there is significant damage to the plasma membrane (13,17,21,33,34,35,37,45,57,58). While it is not known whether this damage to the plasma membrane occurs during freezing (8,46,58,Chapter 4) or thawing (20,33,34), or whether IIF is the cause of (21,34,35) or results from (8,46,58) this damage, the fact is that damage to the plasma membrane is almost always observed after IIF (13,17,21,34,35,37,45,57,58). Regardless of the source of damage, the ensuing rupture of the plasma membrane would invariably be lethal.

The response of single attached cells to intracellular ice formation was consistent with the current concept that intracellular ice formation is lethal (13,37,38,59). In the V-79W and MDCK single attached cells there was a direct correlation between the formation of intracellular ice, the rupture of the cell plasma membrane and lethal cell damage. In this case, damage to the plasma membrane would serve as an excellent predictor of cell survival after IIF. The damage to the cell plasma membrane in the single attached cell model is consistent with the current theories on the involvement of the membrane in IIF (16,26,32,45).

The response of V-79W and MDCK confluent monolayers to intracellular ice formation was significantly different than that of the single attached cells. In most of the cells in this experimental system, the plasma membrane was not damaged during the formation or melting of intracellular ice. The same experimental samples also displayed a similar degree of preservation of metabolic activity and clonogenic function following intracellular ice formation. Statistically, there was no difference in the incidence of membrane damage compared to metabolic activity and clonogenic function. This indicates that those cells that display an intact plasma membrane are the same cells that are able to reduce the alamarBlue reagent and form colonies. Together this data demonstrates that, regardless of the presence of intracellular ice, if membrane integrity can be maintained, then cells will not be lethally injured.

In this study we have observed the unique situation where intracellular ice formation can occur without membrane damage. By performing post-thaw assessments on these experimental samples, a strong correlation between membrane damage and cell injury was made. We conclude then, that maintenance of membrane integrity is critical to the recovery of biological function following intracellular ice formation.

Formation versus Presence of Intracellular Ice

The data from this study provides an opportunity to examine the relationship between intracellular ice and cell death. In single attached cells there is a strong correlation between the formation of intracellular ice, a loss of membrane integrity and cell death. However, in the confluent cell monolayers, some IIF is innocuous, resulting in an intact plasma membrane and the preservation of metabolic activity and clonogenic function. Plasma membrane integrity is an important determinant of lethal damage and not the presence of intracellular ice. This is the first evidence presented that directly uncouples the presence of intracellular ice and lethal, cell damage. Attempts to link intracellular ice, membrane damage and cell viability have eluded previous researchers (16,35).

As intracellular ice is not by itself inherently lethal, then it is the mechanism by which intracellular ice forms that may cause lethal cell damage. Since in confluent cell monolayers IIF can occur without the disruption of the cell plasma membrane, then the mechanism by which intracellular ice forms in this system must be different than that which occurs in the single attached cells where the cells are damaged. Lethal cell injury occurs if the mechanism by which intracellular ice is nucleated within a cell results in the disruption of the plasma membrane.

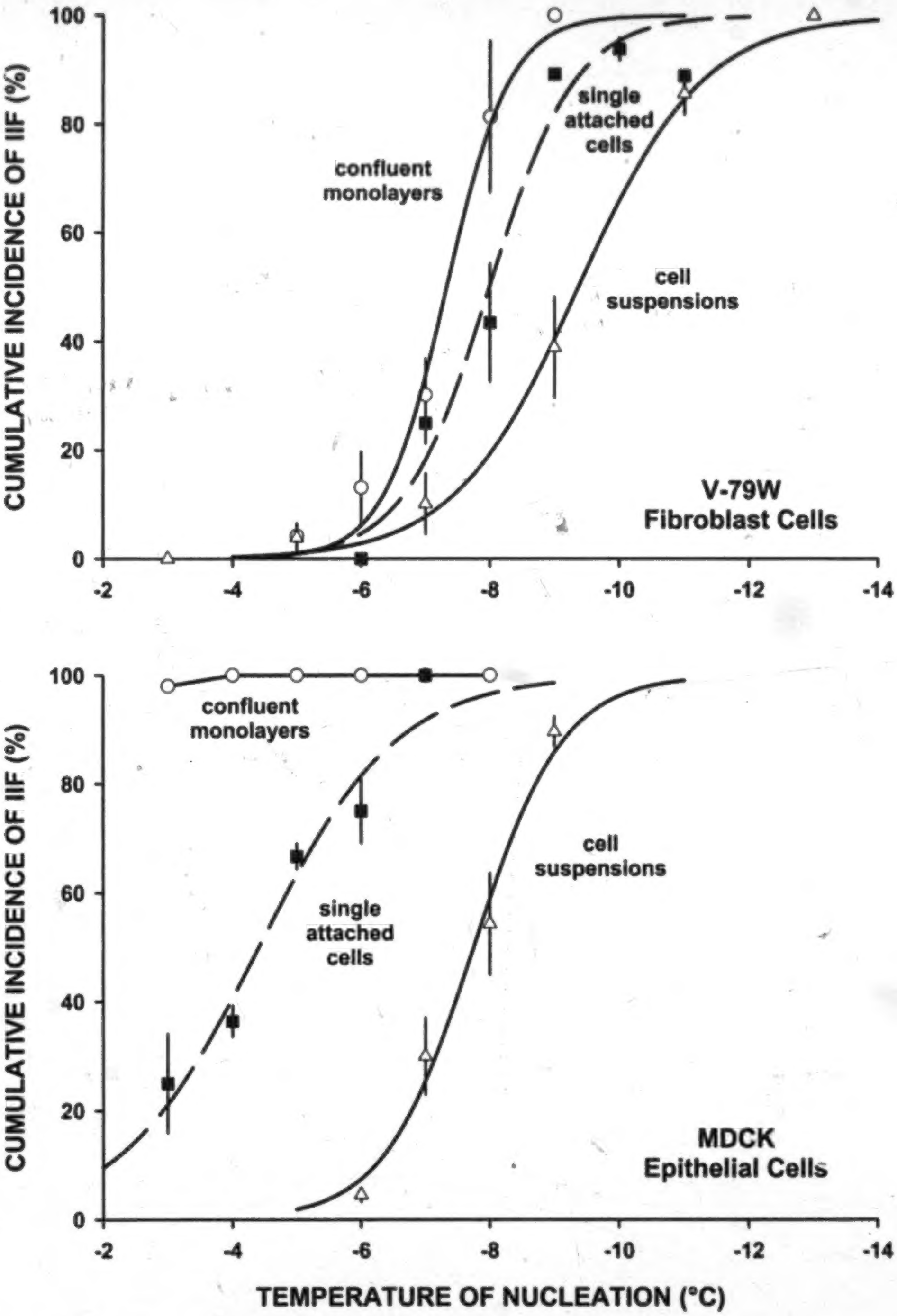

Figure 2.1 – Cumulative incidence of intracellular ice formation in V-79W and MDCK tissue model systems. Reproduced with permission from Acker (1).

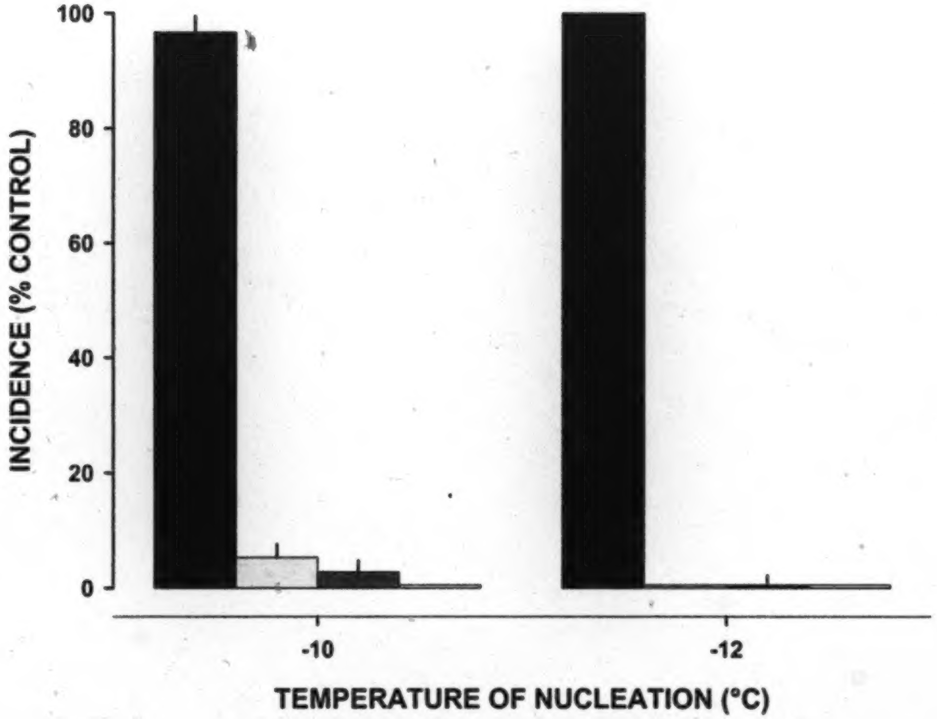

Figure 2.2 – Effect of intracellular ice formation on the post-thaw survival of V-79W single attached cells. The incidence of intracellular ice formation (■), membrane integrity (▨), metabolic activity (■) and clonogenic function (□) is plotted as a function of nucleation temperature. Mean ± SEM (n=6).

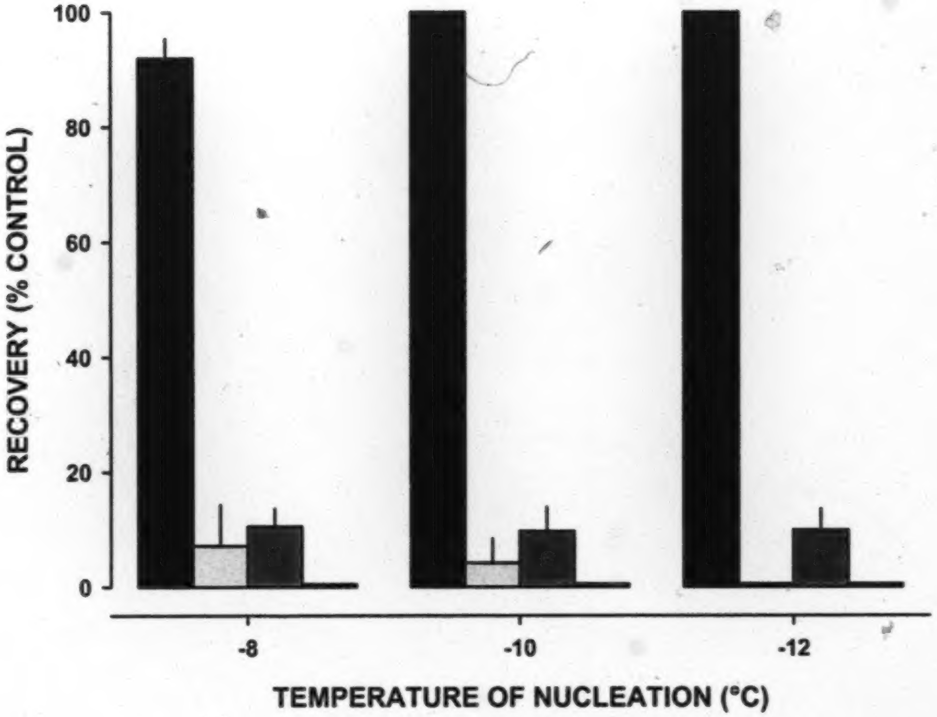

Figure 2.3 – Effect of intracellular ice formation on the post-thaw survival of MDCK single attached cells. The incidence of intracellular ice formation (■), membrane integrity (▨), metabolic activity (■) and clonogenic function (□) is plotted as a function of nucleation temperature. Mean ± SEM (n=6).

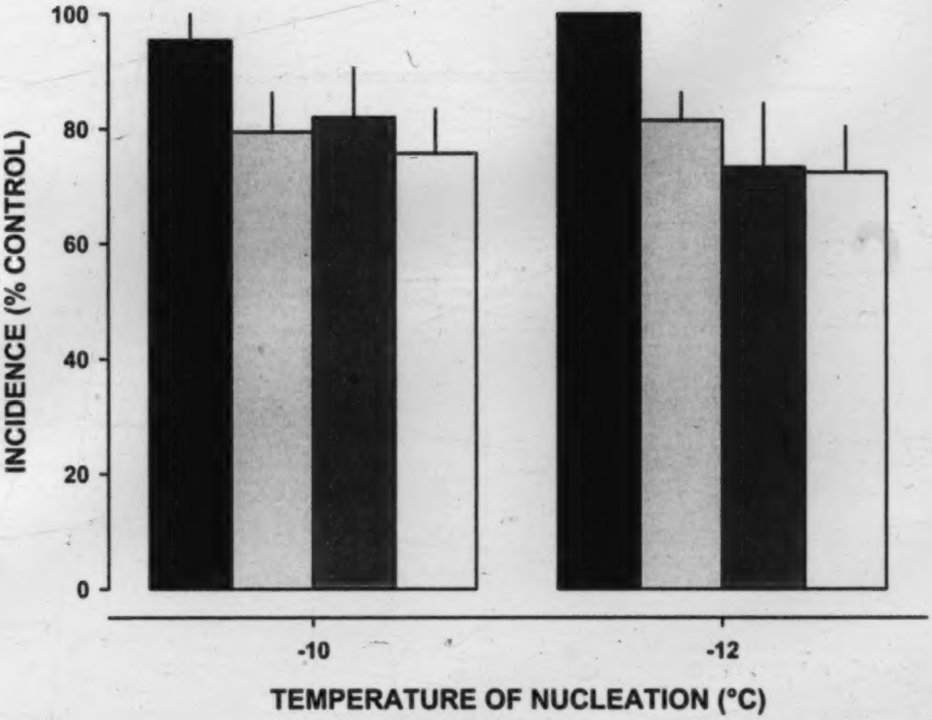

Figure 2.4 – Effect of intracellular ice formation on the post-thaw survival of V-79W confluent monolayers. The incidence of intracellular ice formation (■), membrane integrity (▨), metabolic activity (■) and clonogenic function (☐) is plotted as a function of nucleation temperature. Mean ± SEM (n=6).

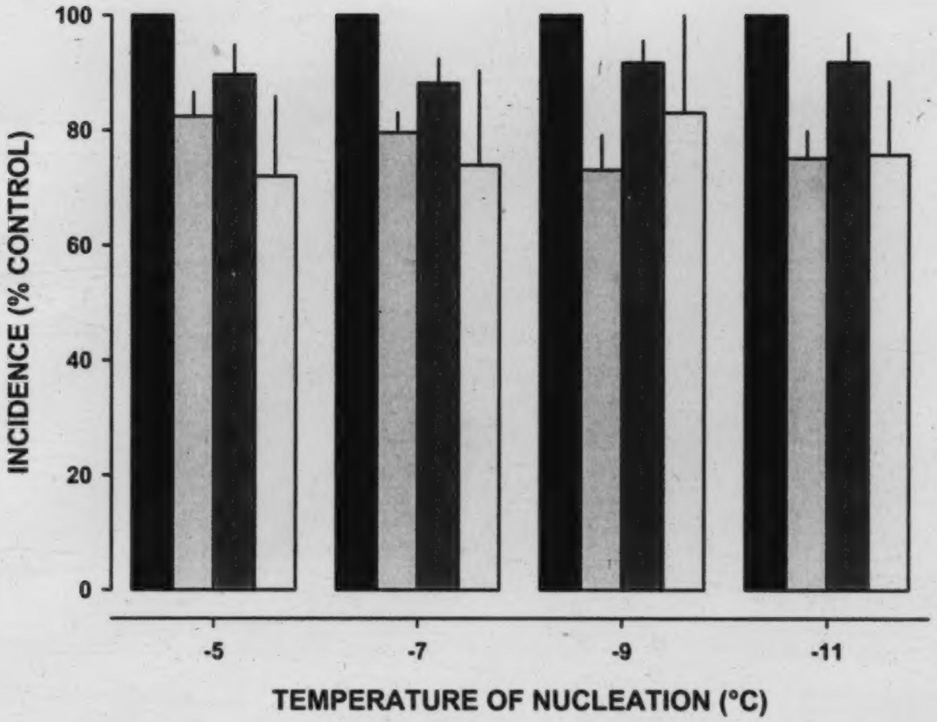

Figure 2.5 – Effect of intracellular ice formation on the post-thaw survival of MDCK confluent monolayers. The incidence of intracellular ice formation (■), membrane integrity (▨), metabolic activity (■) and clonogenic function (□) is plotted as a function of nucleation temperature. Mean ± SEM (n=6).

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CHAPTER THREE
INTRACELLULAR ICE FORMATION AND THE BIOLOGICAL RESPONSE TO FREEZING

3.1 INTRODUCTION

Understanding the freezing response of tissues is an essential step in the development of successful cryopreservation protocols. Biophysical studies have provided a detailed understanding of the biological freezing response of cell suspensions, but efforts to adapt this understanding of cell cryobiology to tissues and tissue model systems have been largely unsuccessful. The failure of this approach is likely a result of the complexity of organized tissues and the lack of information available on the tissue-specific elements involved in freezing.

The response of cells to freezing is critically dependent on the cooling and warming rates that are used. Cell damage during freezing has been attributed to two factors that are directly related to the cooling rate (26). During slow cooling, the cell is dehydrated as the extracellular solutes are concentrated by removal of pure water (ice) from the solution. It has been proposed that damage results from the excessive dehydration of the cell and the increased intracellular and extracellular solute concentrations (20,26,31). During rapid cooling, the formation of extracellular ice and the concentration of solutes occur too quickly for the cell to respond by exosmosis, resulting in the cytoplasm becoming increasingly supercooled with a concomitant increase in the probability of freezing. There is strong evidence to suggest that the formation and/or presence of intracellular ice is lethal to cells (9,17,21,25). In cell suspensions, cooling a sample too slowly or too rapidly will have detrimental effects on post-thaw survival. It has been assumed that the freezing response of tissue systems follows this two-factor model of cryoinjury (18).

The unique physical properties of tissues have been shown to significantly effect the biological response to freezing. The diversity of cell types and cell density as well as the morphological differences between constituent cells determines the osmotic and thermal state of a tissue (18,35), which influences the cooling rates that can be attained and hence the response of the tissue to freezing and thawing. In addition, cell-cell and cell-matrix interactions in a tissue have been implicated in the poor survival of tissues after freezing (4,5,7,14,41), and the presence of cell junctions has been shown to significantly reduce the optimal cooling rate for the preservation of tissue function (6,8,13,14). It has been proposed that these effects are the result of an increased incidence of intracellular ice formation (6,8,13,14). IIF has been shown to be significantly affected by the presence and type of cellular junctions (1,2,7,36).

Intracellular ice formation has been shown to be an important element in the cryopreservation of tissues. Not only are the constituent cells of a tissue more susceptible to the formation of intracellular ice (1,2,6,7,8,13,14,36), but the degree of cell damage is related to the mechanism of ice formation (3, chapter 2). As intracellular ice can form within a cell and remain innocuous (chapter 2), the presence of this ice could have important implications in the response of the cell to further cooling and warming. As it may be difficult to prevent the formation of intracellular ice in tissues, and in some cases IIF may be desired, it is important that there be a basic understanding of the freezing response of tissues in the presence of intracellular ice.

The purpose of this study is to examine the effect of intracellular ice formation on the response of two tissue model systems to freezing; one where single attached cells adhere to a substrate without cell-cell interactions, and the other a confluent monolayer.

3.2 MATERIALS AND METHODS

Cell Culture

Two cell lines were used to investigate the effect of intracellular ice formation on the freezing response of confluent monolayers and single attached cells. The first was the Madin Darby Canine Kidney (MDCK; CCL34 ATCC) epithelial cell line. Cells were grown in tissue culture flasks (25 cm²; Corning Glass Works, Corning, NY) in minimum essential media (MEM) supplemented with 10% v/v fetal bovine serum (all components from GIBCO Laboratories, Grand Island, NY) at 37°C in an atmosphere of 95% air + 5% carbon dioxide. Cells were harvested by exposure to a 0.25% trypsin-EDTA solution (GIBCO) for 10 min at 37°C and were re-suspended in supplemented MEM to obtain cell suspensions and then plated on sterilized cover slips (12 mm circle, Fisher Brand) at a concentration of $2x10^4$ cells/mL. The cover slips were kept in petri dishes in an incubator for 12 h to allow the cells to attach (single attached cells) and 3 d to allow the growth of a confluent monolayer.

The second cell line was the V-79W line of Chinese hamster fibroblasts. Cells were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential medium (MEM) with Hanks' salts, 16 mmol/L sodium bicarbonate, 2 mmol/L L-glutamine and 10% fetal bovine serum supplemented with antibiotics (penicillin G (50 units/mL), streptomycin (50 µg/mL)) (all components from GIBCO Laboratories, Grand Island, NY). Cells were grown in tissue culture flasks (25 cm^2; Corning Glass Works) and harvested by exposure to a 0.25% trypsin solution (GIBCO) for 10 min at 37°C. The fibroblasts were resuspended in supplemented MEM. Sterilized cover slips (12 mm circle, FISHER Brand) were placed in a petri dish (FISHER Brand, 100 x 15 mm) and covered with 15 mL of supplemented MEM containing $3x10^5$ cells. The petri dishes were incubated for 12 h to allow the cells to attach (single attached cells) and 3 days to allow the growth of a confluent monolayer.

Graded Freezing Protocol

Confluent monolayers and single attached cells were placed in glass tubes (15 x 45 mm, Kimble Glass Ltd.) with 50 µL of tissue culture media and immersed in an alcohol bath (Multi-Cool, FTS Systems Inc., Stone Ridge, NY) pre-set at –5°C. Ice formation was induced in the samples using cold forceps, and the samples were held at –5°C for five minutes for the dissipation of the latent heat of fusion. The bath was then cooled at 1°C/min, and at different subzero temperatures (-5, -10, -15, -20, -30, -40°C), samples were either thawed directly in a water bath at 37°C and assessed, or plunged directly into liquid nitrogen (-196°C) for 20 min before rapid thawing in a water bath at 37°C and then assessed. A schematic of the graded freezing protocol is shown in Figure 3.1

In a separate set of experiments, the temperature at which the samples were nucleated was lowered to –10°C to increase the incidence of intracellular ice formation at this lower nucleation temperature. Samples were placed in 5 x 45 mm glass tubes and immersed in an alcohol bath at –5°C. The bath was then cooled at 1°C/min and at –10°C extracellular ice was induced in the samples using cold forceps and the bath was then held at –10°C for 5 min. The bath was then cooled at 1°C/min and the samples treated as before.

This technique has been demonstrated to be a useful analytical technique for progressively simulating cell injury during freezing (27,28,30).

Assessment Techniques

Membrane Integrity: A dual fluorescent staining technique was used for the quantitative assessment of the integrity of the cell plasma membrane. This procedure is described in detail elsewhere (Appendix A). SYTO 13, a permeant live cell nucleic acid dye (1.25 µM) and ethidium bromide (EB; Sigma Chemical Company, Mississuaga, ON; 2.5 µM) where used to differentially stain the cells. Percent survival based on membrane integrity was calculated as the number of SYTO positive cells over the total number of cells (SYTO and EB positive) using equation A-1.

Metabolic Activity: AlamarBlue™ (Biosource International, CA) was used to assess the overall metabolic activity of the confluent monolayers and single attached cells post-thaw (Appendix A). AlamarBlue was added to the tissue culture media (5% v/v) and confluent monolayers (MDCK and V-79W) were incubated in this solution for 12 hours at 37°C. Single attached cells (MDCK and V-79W) were incubated for 24 hours at 37°C in 5% alamarBlue. Following the incubation, an aliquot (100 µL) of the media was removed and measured on a spectrophotometer (570-600 nm; UVmax, Molecular Dynamics, CA). Percent survival based on metabolic activity was calculated as the mean percent difference in reduction between the experimental samples and the controls using equations A-2 and A-3.

Measurement of Temperature Profile in Bulk Systems

For comparison between data gathered using the cryomicroscope (chapter 2) and the graded freezing protocol it was necessary to validate the biological freezing response for bulk freezing protocols. The temperature profile for confluent monolayers and single attached cells were monitored using a calibrated 0.0127 cm copper-constantan thermocouple (OMEGA Engineering Inc., Stanford, CT) and a computer-controlled interface (DAQ-Book, OMEGA Engineering Inc.). The data was correlated with observations on the formation of intracellular ice. The formation of intracellular ice was determined post-thaw using a previously described, fluorometric method (1,3). The effect of changing the fluid volume placed overtop the confluent monolayers and single attached cells on the incidence of IIF and temperature profile was determined (0 μL to 500 μL).

Statistical Analysis

Unless otherwise indicated, a one way ANOVA was used with a level of significance set at 0.05.

3.3 RESULTS

Factors Affecting IIF in Bulk Systems

A typical temperature profile for the graded freezing of a sample with 500 μL of suspending media is shown in Figure 3.2. One experimental sample was plunged into liquid nitrogen after nucleation at –5°C and then thawed after approximately 10 min, and the other sample was rapidly warmed following ice nucleation. The region that was of particular interest to our study was the section where ice was nucleated. This critical region is expanded in Figure 3.3 for different sample volumes.

With a fluid volume of 500 μL, the dissipation of the latent heat of fusion required approximately 5 min. By reducing the volume of fluid on top of the cover slips, there was an accompanying decrease in the time required to dissipate the latent heat of fusion. For fluid volumes of 250, 50 and 0 µL the time required was 2.5, 0.75 and <0.5 min respectively.

IIF has been shown to be dependent on the degree of supercooling across the cell plasma membrane (19,24). It is of interest, then, to know if fluid volume, and hence thermal profile, has an effect on the formation of intracellular ice during bulk freezing. In Figure 3.4 photomicrographs are shown of two different V-79W confluent monolayers cooled to –10°C and then nucleated. According to previous work (Figure 2.1), nucleation at –10°C on a cryomicroscope results in complete formation of intracellular ice. With 500 µL of tissue culture media on top of the cover slips (Figure 3.4A) there was a low incidence of intracellular ice (~10%). However, by reducing the volume of fluid to 50 µL, 100% IIF was observed (Figure 3.4B). Similar results were obtained for MDCK single attached cells and confluent monolayers and V-79W single attached cells (data not shown).

When no media was added to the cover slips, there was a dramatic increase in the disruption of the plasma membrane integrity seen post-thaw (data not shown). This occurred as a result of sample dehydration during the freezing and post-thaw assessment.

Freezing Response of Single Attached Cells and Confluent Monolayers

The survival of V-79W and MDCK single attached cells and confluent monolayers following graded freezing are shown in Figures 3.5 to 3.8.

For V-79W fibroblast cells there is a significant decrease in the survival of single attached cells (Figure 3.5) compared to the survival of confluent monolayer (Figure 3.6) (p=0.034) at low subzero temperatures. V-79W single attached cells that are thawed directly from an experimental temperature display a gradual decrease in both metabolic activity and membrane integrity, with a marked change at –20°C. Confluent monolayers follow a similar pattern. The survival of confluent monolayers that are plunged into liquid nitrogen from the experimental temperatures are substantially greater than the survival of single attached cells.

MDCK epithelial cells display a much larger difference between the survival of single attached cells (Figure 3.7) and confluent monolayers (Figure 3.8). Both the metabolic activity and membrane integrity of MDCK single attached cells that are thawed directly from the experimental temperature rapidly decrease between 0 and –10°C. MDCK confluent monolayers exhibit significantly greater survival following direct thaw than single attached cells ($p<0.001$). There was little difference between the survival of MDCK single attached cells and confluent monolayers that were plunged into liquid nitrogen prior to rapid warming.

Effect of IIF on Freezing Response

To induce a greater degree of intracellular ice formation in single attached cells and confluent monolayers, the temperature of nucleation was decreased from –5 to –10°C. According to Figure 2.1, with 10°C of supercooling, both V-79W and MDCK single attached cells and confluent monolayers should display 100% IIF. This was verified by photomicrographs such as those in Figure 3.4. Metabolic activity and membrane integrity of V-79W and MDCK single attached cells and confluent monolayers following IIF and graded freezing are shown in Figures 3.9 to 3.12.

IIF correlated with the survival of V-79W single attached cells (Figure 3.9) compared to confluent monolayers (Figure 3.10) ($p=0.004$). V-79W single attached cells (thaw direct) displayed a rapid decrease in survival following nucleation at –10°C. Confluent monolayers that were thawed directly from the experimental temperatures maintained a high degree of post-thaw survival (~50%).

The freezing response of MDCK single attached cells (Figure 3.11) is significantly affected by the formation of intracellular ice compared to confluent monolayers (Figure 3.12) ($p<0.001$). The metabolic activity and membrane integrity of MDCK single attached cells displays a dramatic decrease following nucleation at –10°C. In contrast, MDCK monolayers display greater than 60% post-thaw survival following direct thaw.

A large proportion of the constituent cells in V-79W and MDCK confluent monolayers survived the plunge to liquid nitrogen (~40%). This is significantly greater than the survival observed in confluent monolayers that were nucleated at –5°C (V-79W p=0.049, MDCK p=0.029).

Comparing nucleation at –5 and –10°C indicates that the complete formation of intracellular ice significantly reduced the survival of V-79W and MDCK single attached cells. While there was some survival of V-79W and MDCK single attached cells following nucleation at –5°C, there was very little survival following nucleation at –10°C. This is consistent with data presented in chapter 2 indicating that intracellular ice formation is lethal to single attached cells.

Reducing the nucleation temperature and increasing the incidence of intracellular ice formation enhanced the post-thaw survival of V-79W and MDCK confluent monolayers. V-79W confluent monolayers displayed a slightly greater degree of survival when nucleated at –10°C. Reducing the nucleation temperature resulted in a significant increase in the survival of MDCK confluent monolayers (p=0.029).

3.4 DISCUSSION

Fluid Volume Affects Incidence of IIF in Bulk Freezing Systems

Moving from the cryomicroscope to bulk freezing devices requires careful consideration of the differences that exist between the two systems and the effects on the freezing response of the biological material. The microscopic environment of the cryomicroscope allows precise control of the thermal history of a sample. Fluctuations in sample temperature as a result of the latent heat of fusion during freezing or thawing are offset by the high cooling and heating capacity of the stage. However, in bulk systems, geometric considerations become much more important in regulating the temperature profile of a sample. Freezing and thawing events can result in significant deviation from a desired cooling protocol (12). These changes will affect the freezing and thawing environment and hence will influence conditions to which cells and tissues must respond.

To compare data from cryomicroscopy and bulk freezing experiments, it is important to verify that the single attached cells and confluent monolayers respond in a predictable manner. The formation of intracellular ice in single attached cells and confluent monolayers was critically dependent on the volume of fluid surrounding these experimental systems. By modulating the fluid volume it was possible to reduce the time required for the dissipation of latent heat and increase the incidence of intracellular ice. To replicate the incidence of intracellular ice observed using cryomicroscopy (Figure 2.1) required that there be very little fluid on top of the samples (50 µL). With increasing fluid volume, the latent heat generated during freezing raises the sample temperature and inhibits the formation of intracellular ice.

The effect of fluid volume on the biological response of a variety of tissues has been reported (10,15,16). The beneficial effect of cryopreserving tissue in the absence of media has been best characterized in the cornea (10,11,30,33,37) where minimizing the volume of the cryopreservation solution significantly improves tissue survival (10,11,33,37). This effect has been attributed to the change in extracellular ice distribution within the cornea that prevents damage to the endothelial cells and Descemet's membrane (10). The data presented here suggests an alternate explanation -- that cryopreserving tissue in the absence of extracellular media has an important effect on the temperature profile of the sample and hence the incidence of intracellular ice formation in a confluent monolayer.

Investigation of Cryoinjury Using Graded Freezing

The graded freezing protocol is an experimental tool that gives researchers the means to examine the effects of freezing on the biological response of cells and tissues. From a single experiment, the effects of slow and rapid cooling on the survival of cells or tissues can be distinguished. The graded freezing protocol has been used extensively to examine low temperature responses of cell suspensions (27,28,29,30). The response of cell suspensions to graded freezing has been correlated with the current understanding of cryoinjury (28,29,30). The direct thaw curve on a graded freezing diagram is an indication of cumulative slow cooling injury. During slow cooling, after ice nucleation in the extracellular solution, there is an increase in the amount of extracellular ice resulting in an increase in the extracellular solute concentration. This gradual increase in solute concentration results in the exosmosis of intracellular water and a decrease in the cell volume. The concentration of extracellular solutes (20,26) and/or the excessive shrinkage of the cell (31,32,38) have been suggested as the means by which cells are damaged during slow cooling. Rapid cooling injury is thought to result from the formation of intracellular ice that occurs when cells are plunged into liquid nitrogen from a high subzero temperature. At high experimental temperatures, there is a considerable amount of intracellular water that can be frozen when the cells are plunged. As cells become increasingly dehydrated by slow cooling to lower experimental temperatures prior to the plunge, then the incidence of IIF decreases, with a corresponding increase in the survival of plunged cells. This is a typical interpretation of the results obtained from graded freezing experiments with cell suspensions.

For tissue systems, where IIF occurs much more readily, care must be taken in interpreting the results from a graded freezing experiment. The temperature of nucleation becomes critical since intracellular ice formation may occur during the initial nucleation of the samples. Once intracellular ice forms, then there is no longer a driving force for the movement of water across the plasma membrane as the amount of extracellular ice increases, so no further cell shrinkage occurs (23,24). Interpretation of the direct thaw curve must therefore consider this factor in an alternative explanation for slow cooling injury. As significant intracellular ice may form during the extracellular nucleation event, damage resulting from a plunge into liquid nitrogen could no longer be attributed to IIF. Again, an alternative interpretation of the data would be required. The effects of increased intracellular ice formation on the low temperature response of single attached cells and confluent monolayers are discussed below.

Survival of Single Attached Cells is Dependent on Membrane Integrity

The data shows a strong correlation between membrane damage and the incidence of intracellular ice. V-79W single attached cells nucleated at –5°C display ~5% IIF (Figure 2.1) and ~95% intact plasma membranes (Figure 3.5) when thawed directly. Similarly, MDCK single attached cells nucleated at –5°C exhibit ~40% IIF (Figure 2.1) with ~65% of the cells displaying an intact plasma membrane (Figure 3.7). Decreasing the nucleation temperature increases the initial incidence of IIF with a direct effect on membrane integrity. The plasma membrane is a targeted site of IIF injury for single attached cells.

Rapidly plunging single attached cells into liquid nitrogen regardless of the experimental temperature, significantly reduces cell survival. In V-79W single attached cells, nucleation at –10°C resulted in complete formation of intracellular ice in all cells (Figure 3.9). Plunging these samples into liquid nitrogen should not increase the incidence of IIF and the degree of cell injury. A low cell survival would be expected, and in fact was observed for these single attached cells (Figure 3.9). However, for the V-79W single attached cells that are nucleated at -5°C, where few cells form intracellular ice at the temperature of nucleation, there is a similar degree of cell damage as was observed following nucleation at -10°C. From work with cell suspensions (29,30), slowly cooling cells to low temperatures following nucleation at –5°C before plunging into liquid nitrogen should result in an increase in cell survival as dehydration of the cell reduces the probability of IIF. However, for single attached cells, there was a low degree of cell survival for all samples plunged into liquid nitrogen from all experimental temperatures. This discrepancy suggests that single attached cells are much more sensitive to rapid cooling injury than cell suspensions. It has been suggested that surface attachment can inhibit osmotic regulation (2,6), render the membrane more fragile to osmotic changes (2) and/or alter the contact angle of extracellular ice with the membrane (2). During rapid cooling, this would result in significant damage to the plasma membrane leading to cell death. In single attached cells, it is possible that rapid cooling injury may not be the result of intracellular ice formation, but an indication of the effect cell attachment has on membrane integrity during freezing.

The response of single attached cells to slow and rapid cooling is dependent on the presence of an intact plasma membrane. Intracellular ice formation disrupts the plasma membrane of single attached cells, and decreases the survival of cells following freezing and thawing.

Cell-Cell Propagation Enhances Survival of Confluent Monolayers

The survival of V-79W and MDCK confluent monolayers following graded freezing was different from that observed for single attached cells. Two nucleation temperatures were used to examine the effect of intracellular ice formation on the freezing response of confluent monolayers. However, unlike single attached cells, decreasing the nucleation temperature did not decrease the survival of the confluent monolayers. Increasing the incidence of IIF by lowering the nucleation temperature did not affect survival of V-79W cells and increased survival in MDCK confluent monolayers. The survival of confluent monolayers is therefore not directly associated with the presence of intracellular ice.

In V-79W confluent monolayers, decreasing the nucleation temperature results in the incidence of intracellular ice increasing from <10% at –5°C to 100% at –10°C (Figure 2.1). Increasing intracellular ice formation by more than 90%, however, does not result in a proportional effect on the post-thaw metabolic activity (Figure 3.10a) or membrane integrity (Figure 3.10b). As membrane integrity has been shown to be a key indicator of cell viability (chapter 2), increasing the incidence of IIF in V-79W confluent monolayers may not be lethal as a high degree of cells with intact membranes can be recovered. It has been proposed that intracellular ice can be nucleated in adjacent cells without a disruption in the plasma membrane (1,3). At –10°C, the mechanism by which intracellular ice propagates between adjacent cells must dominate over the mechanism whereby intracellular ice formation results in the disruption of the plasma membrane to result in the incidence of membrane integrity observed in V-79W (Figure 3.10b).

In contrast to V-79W fibroblasts, decreasing the nucleation temperature does not affect the incidence of intracellular ice formation in MDCK confluent monolayers. Complete formation of intracellular ice occurs at both nucleation temperatures examined. It has been proposed that the propagation of intracellular ice in MDCK confluent monolayers can occur much more readily than in V-79W due to the presence of gap junctions (1). By facilitating the induction of ice between cells, gap junctions may prevent the disruption of the plasma membrane during intracellular ice formation. The enhanced survival of MDCK confluent monolayers following intracellular ice formation suggests that cell-cell propagation may have an important role in minimizing cell damage.

The biological response of confluent monolayers is significantly different than what is observed in single attached cells and has been reported for cell suspensions (30,39). Confluent cell monolayers are not as readily injured as other cell morphologies when subjected to the graded freezing protocol. In addition, increasing the incidence of intracellular ice formation does not result in a significant decrease in the metabolic activity or membrane integrity. In fact, IIF can increase the survival of confluent monolayers. This is a novel observation and warrants further discussion.

IIF Protects Against Slow Cooling Injury In Confluent Monolayers

During slow cooling, concentration of the intra- and extracellular solutes occurs as a result of the formation of external ice and the efflux of water from the cells (22). Slow cooling injury, or solution effects injury, is thought to occur as a result of this concentration of intra- and extracellular solutes. Damage to cell suspensions has been attributed to two general mechanisms: solute toxicity (20,26), and excessive shrinkage (31,32,38). Solute toxicity remains a relatively undefined mechanism by which cells are injured during slow cooling (24,26). The second mechanism by which slow cooling has been proposed to injure cells is by the shrinkage of the cell that results from exposure to hypertonic solutions. It has been proposed that there is a minimum critical volume that the cell cannot exceed or else damage will result following freezing (31,32). Alternatively, it has also been proposed that the surface area of the cell is reduced as a result of a loss in membrane material during slow cooling (38). Injury occurs as a result of exceeding the cells minimum critical volume, or osmotic rupture due to the reduction in the cells surface area. A recent theory (20,34) suggests that during freezing salt loading occurs which results in the cell exceeding the isotonic volume and osmotically rupturing during thawing. Slow cooling injury is therefore likely a result of the toxic and osmotic events associated with exposure to an increasing concentration of solutes.

The presence of intracellular ice in confluent monolayers partially protects against the damaging effects of slow cooling. In Figure 3.10 and 3.12, the survival of V-79W and MDCK confluent monolayers with extensive intracellular ice remains relatively constant as the samples are slowly cooled. If the confluent monolayers were susceptible to slow cooling injury, then a gradual decrease in survival with decreasing temperature would be expected. As slow cooling injury is evident in the V-79W confluent monolayers nucleated at –5°C, but absent at –10°C, intracellular ice must play an important role.

If slow cooling injury is a result of excessive changes in cell volume during freezing and thawing, then preventing this should protect cells from slow cooling injury. Intracellular ice may protect against solution effects injury by preventing this excessive shrinkage of the constituent cells in the confluent monolayer. In the V-79W and MDCK confluent monolayers, IIF can occur without the immediate disruption of the plasma membrane in a majority of the cells in the monolayer (3). Once intracellular ice forms, then there would no longer be a driving force for the movement of water across the plasma membrane as a result of the increasing extracellular solute concentration (23,24). As the samples are further cooled, more ice will form intracellularly and balance any osmotic gradients that develop across the membrane as a result of extracellular ice formation. Intracellular ice will therefore buffer the effects of increasing solute concentration and prevent the cells from exceeding any minimal cell volume.

In contrast, if slow cooling injury were a result of the osmotic rupture of cells due to the loading of extracellular salt (34), then intracellular ice formation could also prevent this injury. As there is complete formation of intracellular ice in the confluent monolayers during nucleation, there would not be an osmotic gradient to drive the flow of extracellular salt. In addition, the formation of intracellular ice would prevent any excessive cell shrinkage, which has shown to be a requirement for the leakage of salt across the plasma membrane (20,31,40). Intracellular ice would protect cells from salt loading and prevent the cell from exceeding a critical volume following warming.

As more detailed models for slow cooling injury are developed, the role that intracellular ice has in protecting confluent monolayers from damage will evolve. With a better understanding of the protective mechanism of intracellular ice, development of techniques and procedures to eliminate slow cooling injury can be explored.

3.5 SUMMARY

Subjecting single attached cells and confluent monolayers to graded freezing has permitted a detailed examination of the mechanisms by which cells are injured during freezing. Intracellular ice has been shown to be an important element in the degree of cell damage experienced by tissue model systems. Understanding these mechanisms of injury will be critical in the development of novel techniques for the protection of cells, tissues and tissue model systems from cryoinjury.

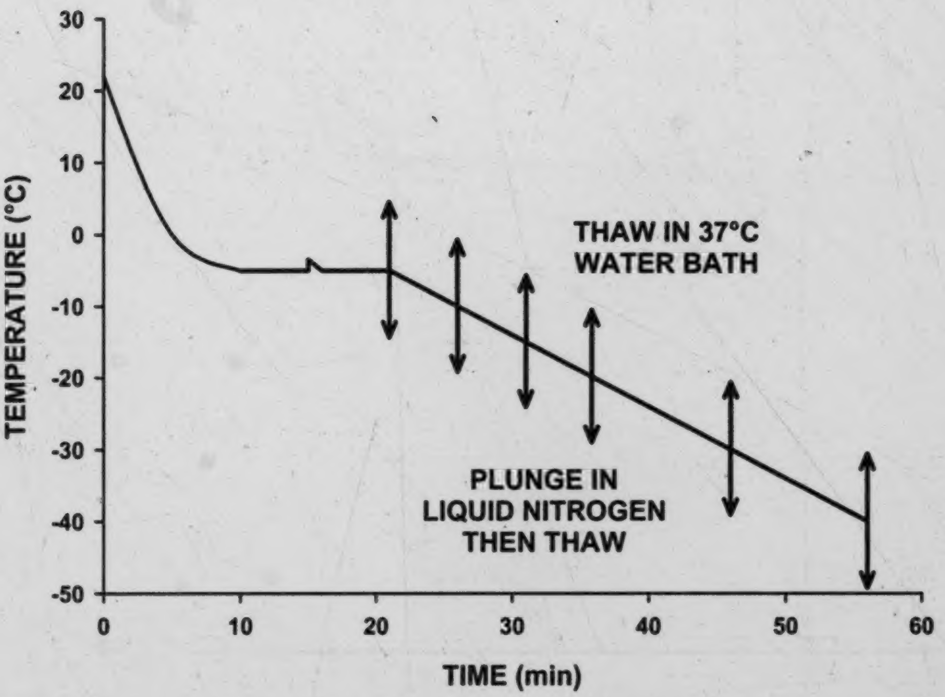

Figure 3.1 – Schematic representation of the graded freezing protocol. This diagram represents samples that would be nucleated at -5°C and then cooled at 1°C/min.

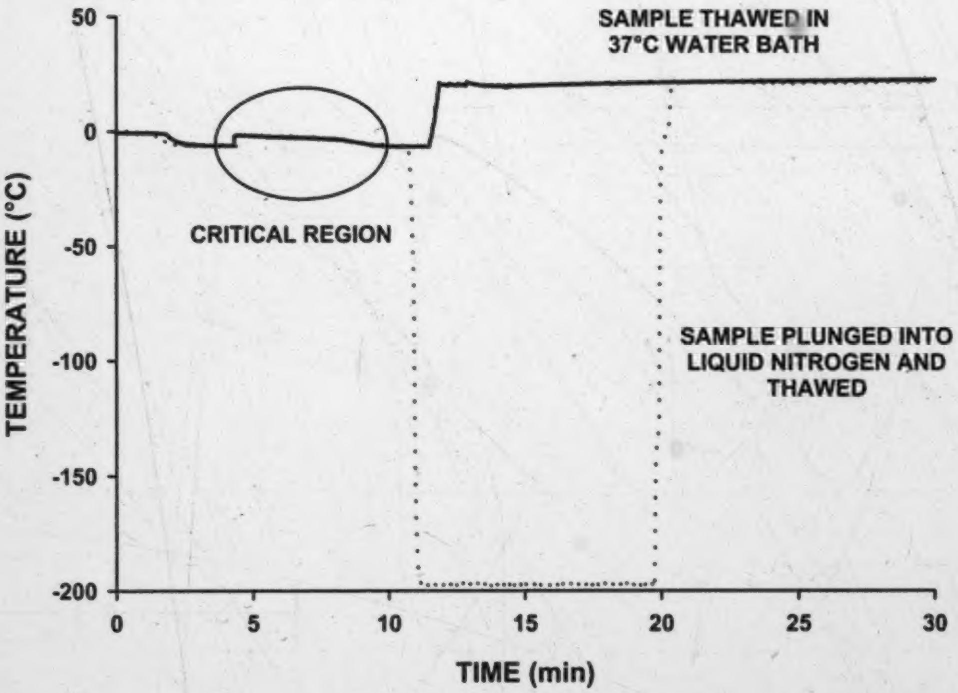

Figure 3.2 – A temperature profile for the graded freezing of a sample with 500 µL of suspending tissue culture media. The samples were nucleated at -5°C and held for 5 min before being cooled at 1°C/min. One sample was plunged into liquid nitrogen after nucleation and then thawed after 20 min, and the other sample was rapidly warmed following extracellular ice nucleation.

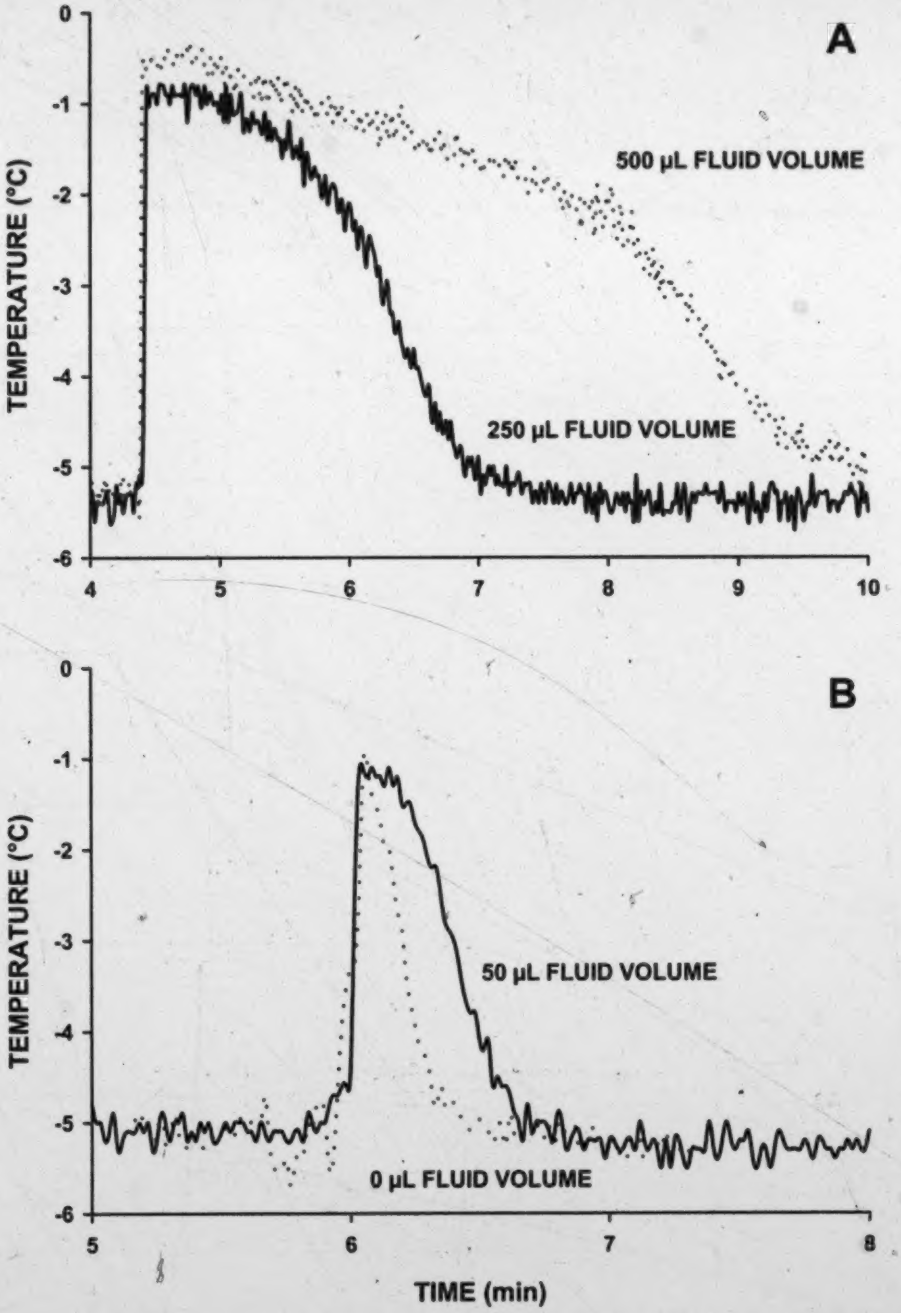

Figure 3.3 – An enlarged view of the nucleation region of the simulated temperature profile for samples in 15 x 45 mm glass vials subjected to a graded freeze. The samples were nucleated at -5°C and held for 5 min before being cooled at 1°C/min. The vials contained either 500 and 250 µL (A) or 50 and 0 µL (B) of tissue culture media.

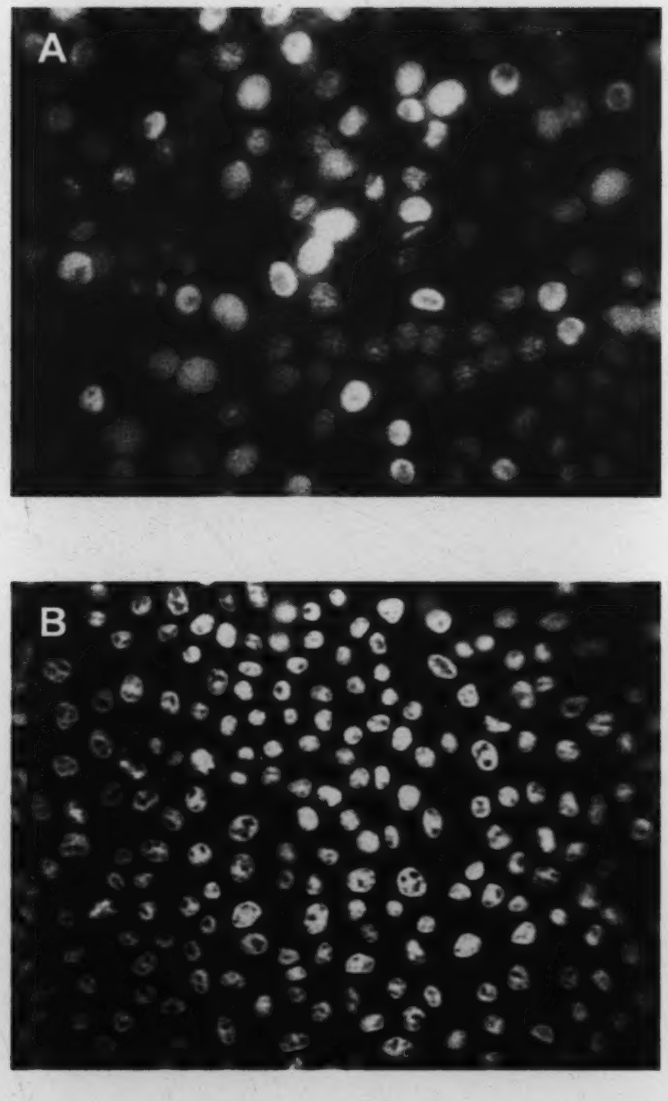

Figure 3.4 – Effect of fluid volume on the incidence of intracellular ice formation in V-79W confluent monolayers nucleated at -10°C. The incidence of intracellular ice formation was much lower with 500 µL of tissue culture media (A) compared to 50 µL of tissue culture media (B).

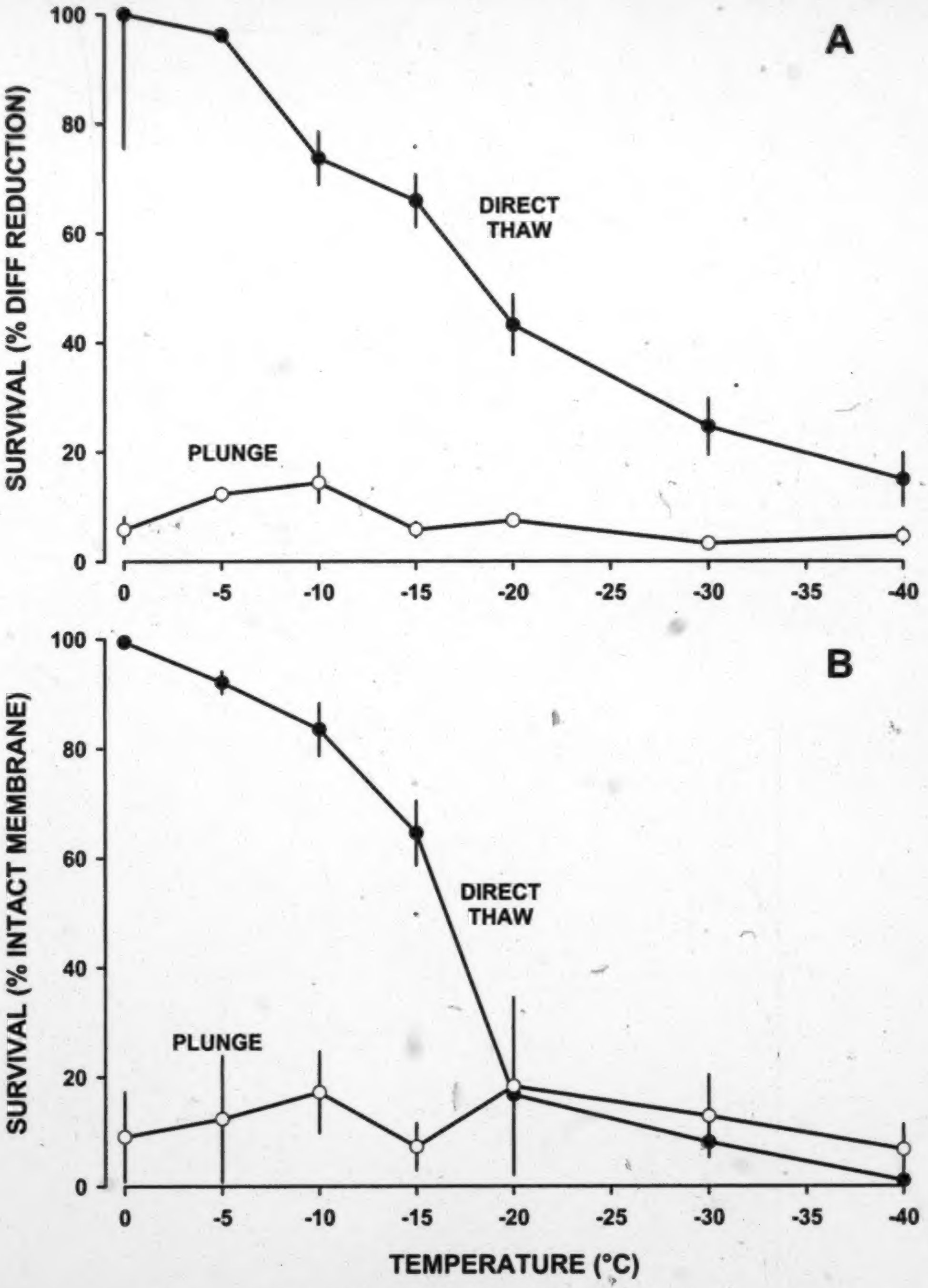

Figure 3.5 – Response of **V-79W single attached cells** to graded freezing. Samples were nucleated at **-5°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

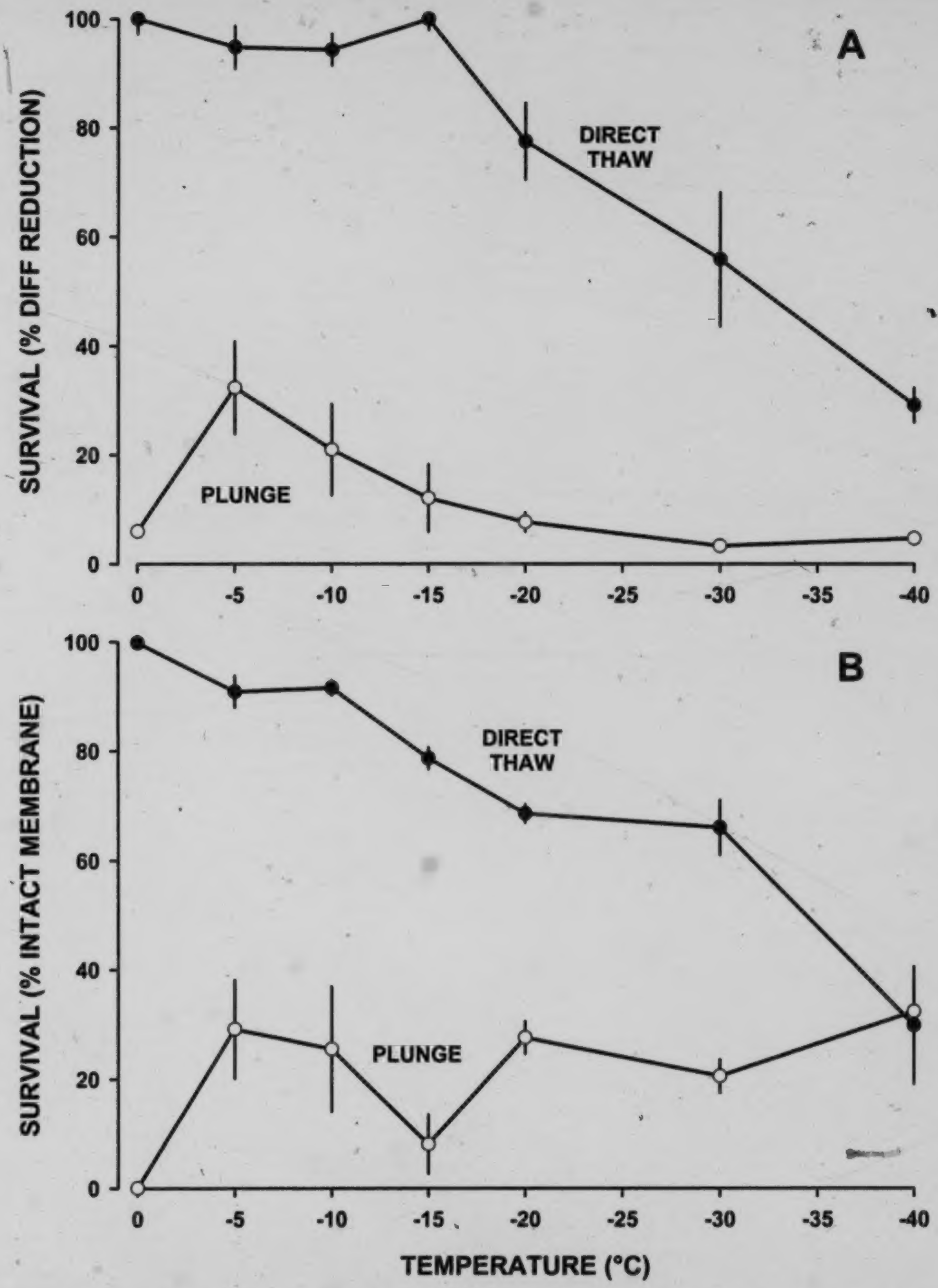

Figure 3.6 – Response of **V-79W confluent monolayers** to graded freezing. Samples were nucleated at **-5°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

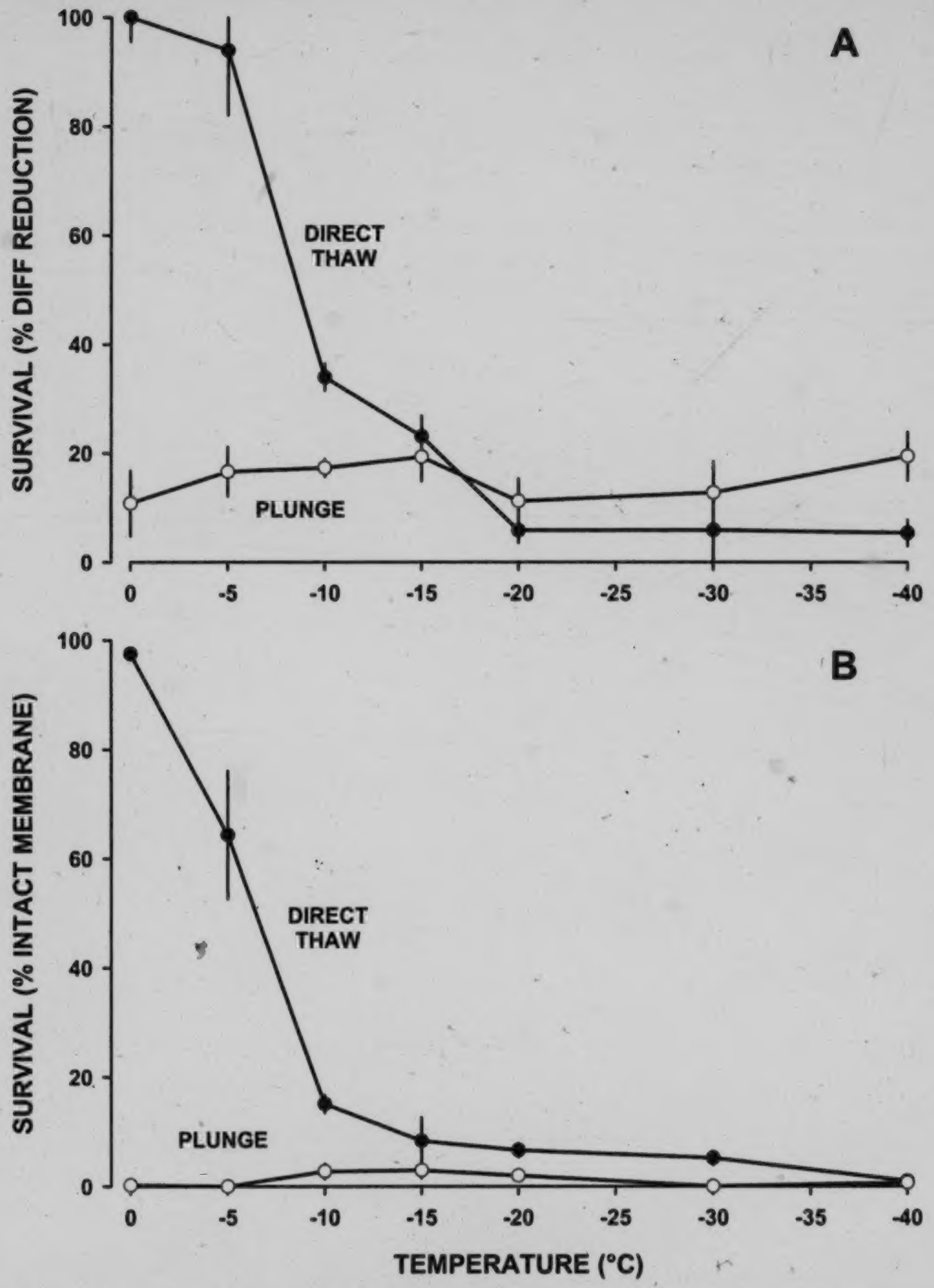

Figure 3.7 – Response of **MDCK single attached cells** to graded freezing. Samples were nucleated at **-5°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

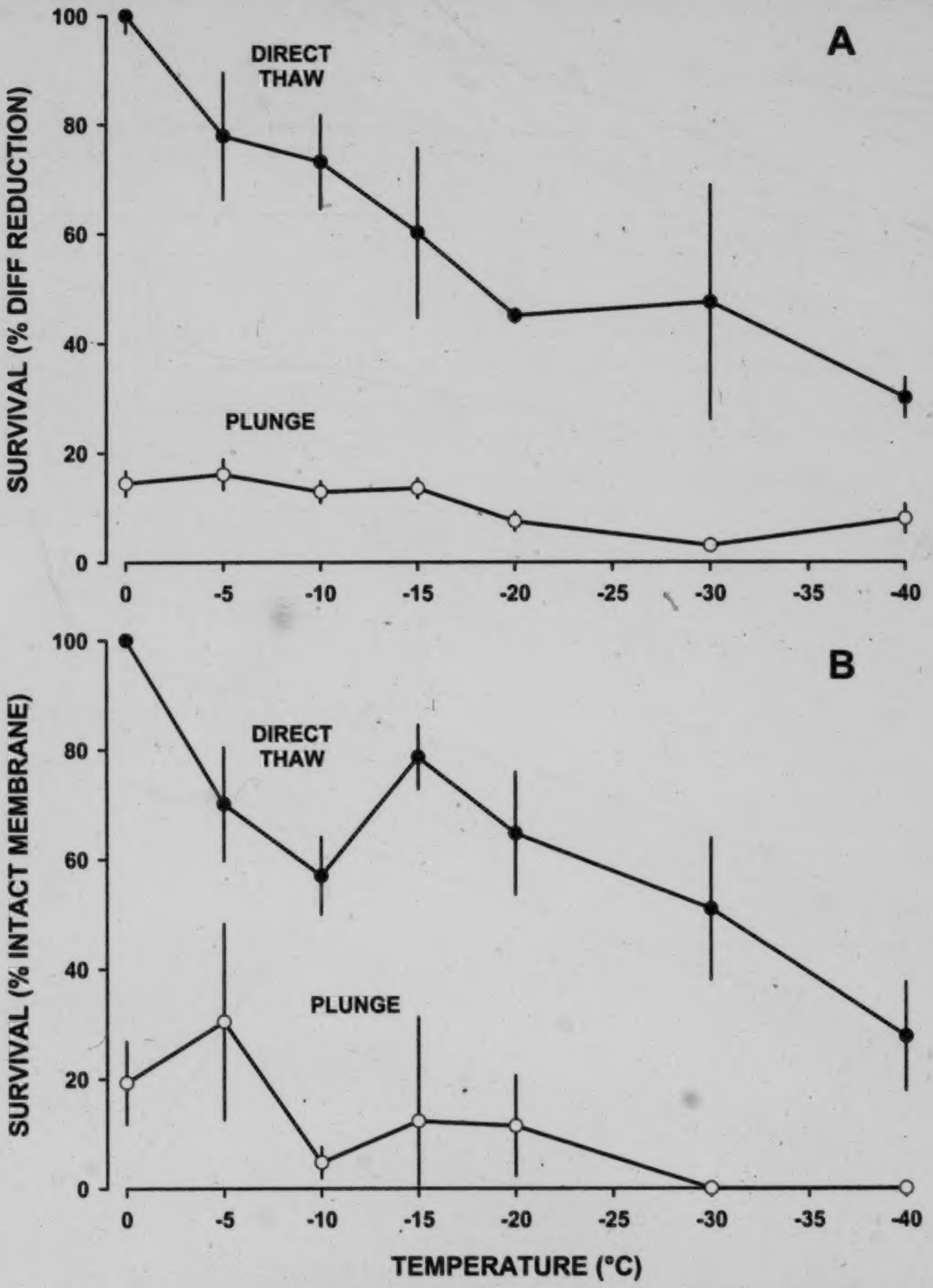

Figure 3.8 – Response of **MDCK confluent monolayers** to graded freezing. Samples were nucleated at **-5°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

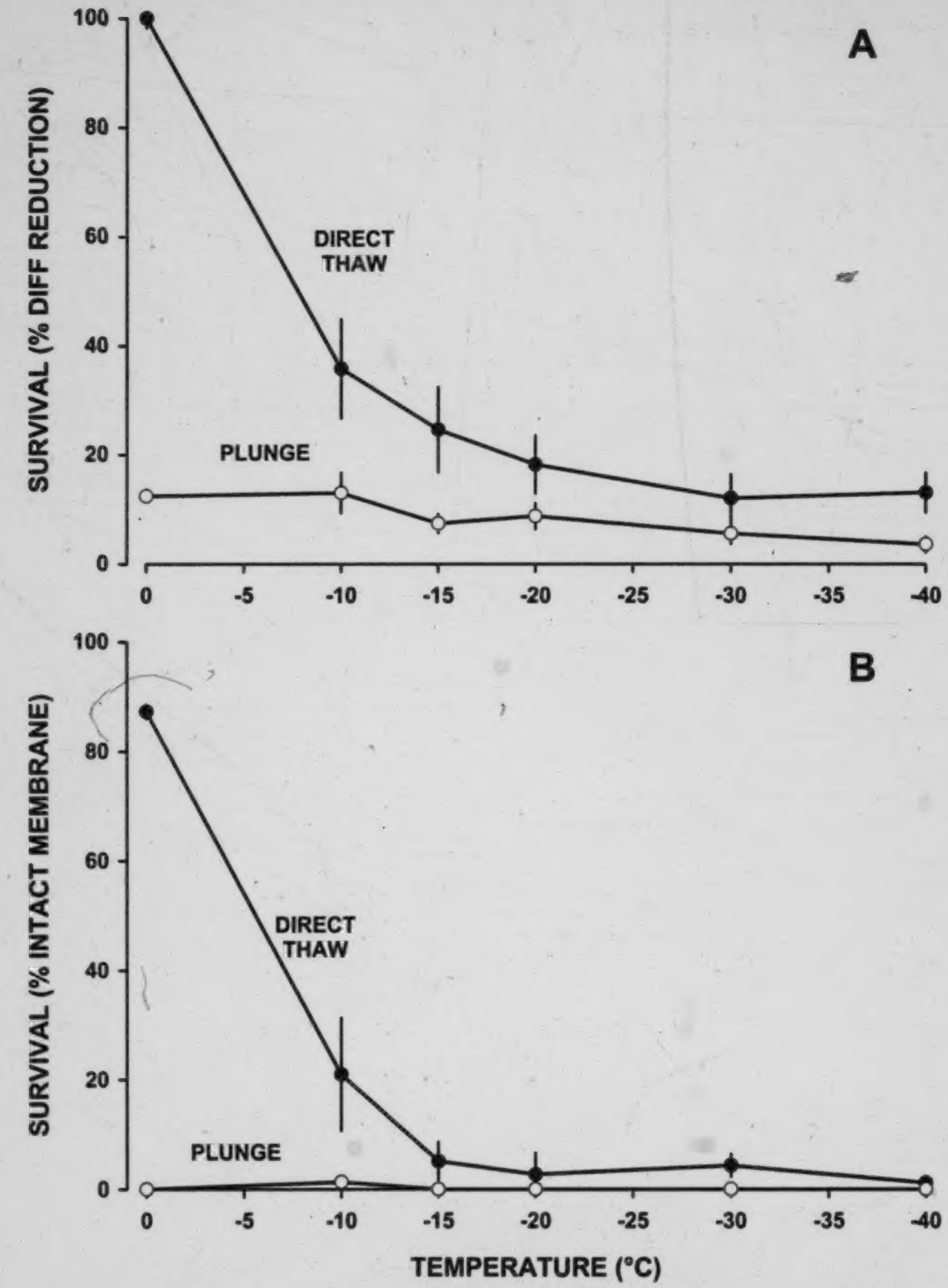

Figure 3.9 – Response of **V-79W single attached cells** to graded freezing. Samples were nucleated at **-10°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

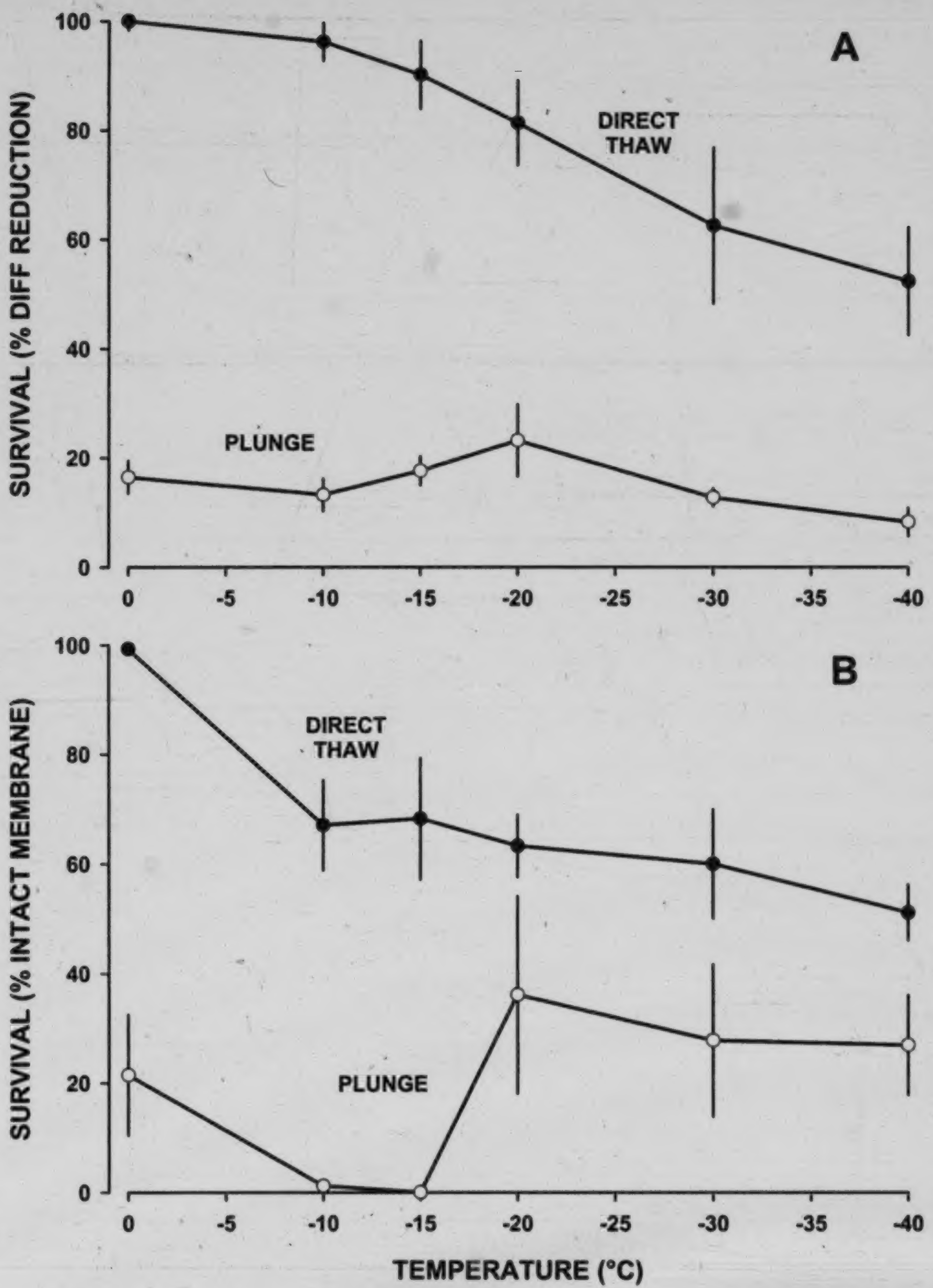

Figure 3.10 – Response of **V-79W confluent monolayers** to graded freezing. Samples were nucleated at **-10°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

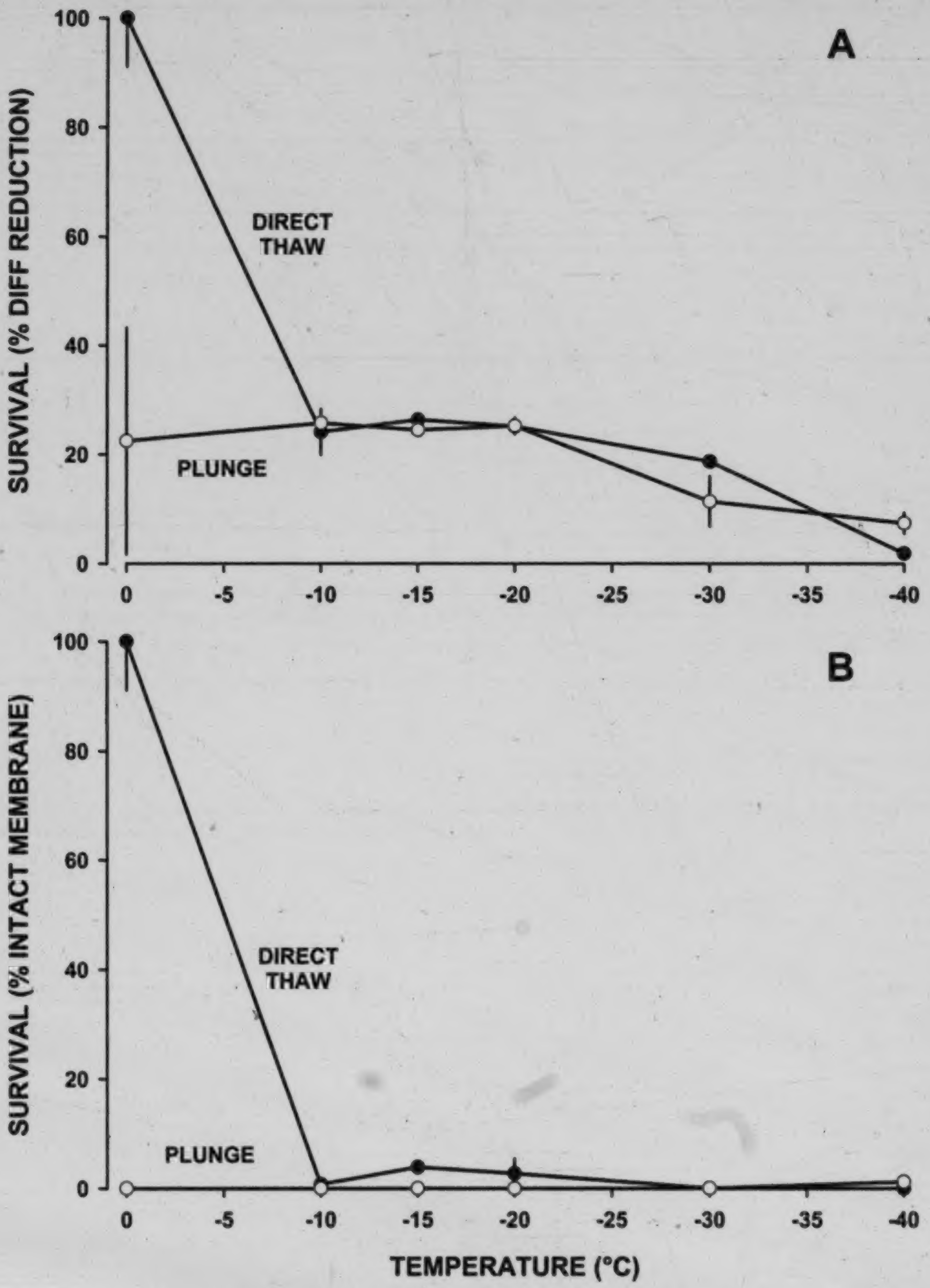

Figure 3.11 – Response of **MDCK single attached cells** to graded freezing. Samples were nucleated at **-10°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

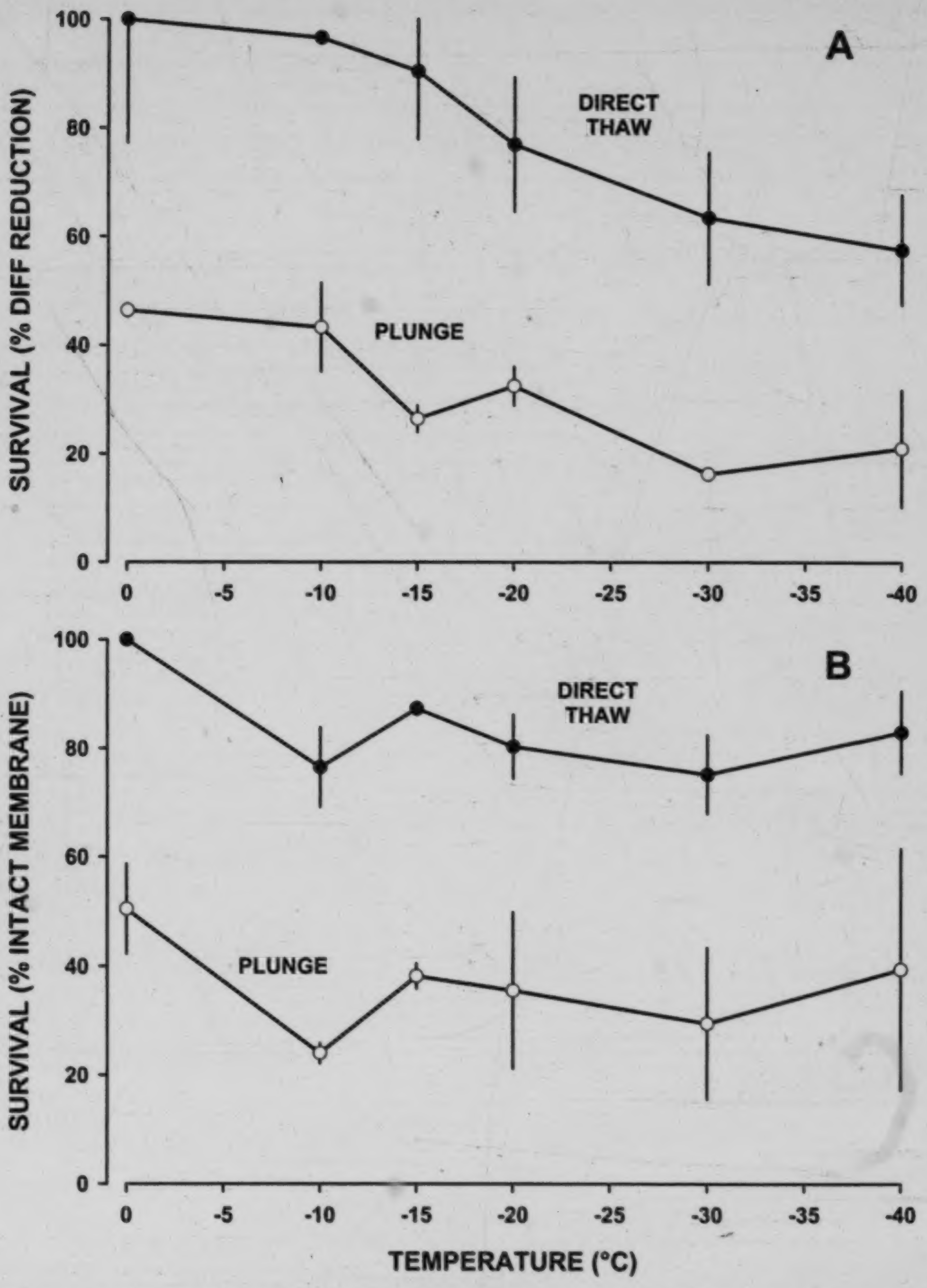

Figure 3.12 – Response of **MDCK confluent monolayers** to graded freezing. Samples were nucleated at **-10°C**, held for 5 min, cooled at 1°C/min and either thawed directly or plunged into liquid nitrogen. The samples were assessed for metabolic activity (A) and membrane integrity (B). Mean ± SEM (n=6).

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CHAPTER FOUR

INTRACELLULAR ICE FORMATION AND THE PLASMA MEMBRANE

4.1 INTRODUCTION

It is generally accepted that there is an intimate relationship between intracellular ice formation (IIF) and the cell plasma membrane (15,19,29,38,39,46). For intracellular ice formation to occur, extracellular ice must cross the semipermeable cell plasma membrane and nucleate a supercooled cytoplasm. The permeability of the membrane to water and solutes dictates the degree of cytoplasmic supercooling that will occur during rapid cooling. As the membrane is a barrier to ice propagation (12,24) and there are few efficient nucleators in a cell (41), nucleation of the supercooled cytoplasm at high subzero temperatures is thought to be the result of extracellular ice (15,19,21,38,39,43,44,46). How this extracellular ice can nucleate the cytoplasm has not been resolved (15,19,38), but clearly, the membrane must be implicated. Determining the relationship between intracellular ice formation and the plasma membrane, therefore, is an important step in developing a comprehensive understanding of intracellular ice formation.

One of the problems that has plagued researchers attempts at understanding the relationship between the plasma membrane and intracellular ice formation is the fact that there is no consensus as to the time course of events surrounding intracellular freezing (15,30). It has been well documented that following intracellular ice formation post-thaw damage to the plasma membrane will be observed (12,16,18,29,30,31,38,43,44,45). It is not known whether this damage to the plasma membrane occurs during freezing (7,39,44) or thawing (17,28,29,30), or whether IIF was the cause of (15,31) or resulted from (7,39,44) this damage. While damage to the plasma membrane is almost universally observed following intracellular freezing, the fact is we do not understand how or when it occurs. Elucidating this temporal sequence of events is critical to developing an accurate model of intracellular ice formation in cells and tissues.

In 1963, Mazur proposed that membrane damaged occurred during thawing, and was the result of intracellular ice formation (28,29,30). As the cell membrane prevents the passage of intracellular solutes, Mazur used the electrical conductivity of a solution to study the integrity of the plasma membrane (28). By monitoring the resistivity of solutions of yeast during warming, he proposed that the temperature at which there was a decrease in resistivity would be the temperature at which membrane damage occurred. Mazur observed a dramatic increase in the electrical conductance (decrease in resistivity) of the yeast solution at –4°C. By comparing his results with yeast to the resistivity of 0.0015 and 0.025 M KCl solutions, Mazur concluded that the cell membrane must be sufficiently intact after freezing to prevent the outflow of intracellular solutes (28). Since 1963, cryobiologists have interpreted this to mean that membrane damage occurs during thawing and not during freezing (15,19,31).

Following his electrical conductivity studies, Mazur looked at the effect of warming rate on rapidly cooled yeast. He observed that if yeast are cooled at rates where intracellular ice formation was known to occur, rapid warming would result in high cell viability (30,32). It is unlikely that these cells could not have been irreversibly damaged during freezing and the formation of intracellular ice as they could be recovered using rapid warming. Yeast cells that were warmed slowly following intracellular ice formation showed a damaged plasma membrane and were dead (30,32). This data was correlated with studies on the ice crystal size during warming, and it was concluded that recrystallization of small ice crystals results in the damage to the plasma membrane and subsequent cell death during slow warming (8,25,26,31,34). The data on yeast cells, therefore, supports the idea that intracellular ice formation results in damage to the plasma membrane and that this damage occurs during slow thawing.

While Mazur's work stands as the accepted sequence of events surrounding the formation of intracellular ice and the disruption of the plasma membrane, it is not without some critics. Disruption of plasma membrane has been proposed as an alternative mechanism by which extracellular ice can nucleate the cell. In this case IIF would be the result of damage already done to the plasma membrane and not the cause of damage as proposed by Mazur. Working with unfertilized eggs of the sea urchin *Hemicentrotus pulcherrimus*, Asahina proposed in 1962 that intracellular ice formation was the result of damage to the plasma membrane (7). By observing the interaction of extracellular ice and salt precipitate with the cell plasma membrane, he concluded that membrane damage precedes IIF. This work parallels the ideas presented in the osmotic rupture hypothesis for freezing injury (39). Motivated by the fact that cells can be damaged at 0°C in the absence of ice at a magnitude of osmotic pressure remarkably similar to those produced during freezing, Muldrew and McGann proposed that membrane damage may be a result of a critical osmotic pressure gradient across the membrane (38,39). While the osmotic pressure gradient itself may not be the damaging factor, the frictional forces developed as a result of the excessive efflux of water may result in rupture of the membrane (39). Disruption of the cell plasma membrane would therefore allow for the entry of extracellular ice into the cell.

The most convincing evidence to support the idea that intracellular ice formation is the result of membrane damage comes from Steponkus and Dowgert, who observed the rupture of membranes of plant protoplasts immediately prior to the formation of intracellular ice (43,44). Using high-resolution cryomicroscopy, they documented the sequence of events leading to the nucleation of the cell cytoplasm and concluded that "mechanical failure of the plasma membrane precedes intracellular ice formation and exposes the supercooled intracellular solution to the external ice matrix" (44). It was proposed that this disruption occurred as a result of the development of electrical transients created at the interface of the growing ice front and the aqueous solution (42). These electrical transients would cause the membrane to burst if they reached a critical magnitude allowing ice from the extracellular solution to nucleate the cytoplasm.

A corollary to the idea that intracellular ice is a result of damage to the plasma membrane is that this damage must occur during freezing. In the work by Asahina (7), Muldrew (39) and Steponkus and Dowgert (44), membrane damage was either observed, or was predicted to occur prior to intracellular nucleation.

While the cause of the membrane damage is unresolved, it must occur prior to or during the freezing process and therefore intracellular ice formation must also take place during freezing.

Understanding the sequence of events surrounding the formation of intracellular ice would significantly enhance our understanding of the response of cells and tissues to rapid freezing (15). As it has been suggested that the maintenance of membrane integrity is critical to the recovery of biological function following intracellular ice formation (Chapter 2 and 3), understanding the sequence of events involved in IIF is crucial in cryobiology. Determining whether membrane damage occurs during freezing or thawing will have important implications in the refinement of the models used to describe the formation of intracellular ice in cells and tissue.

4.2 MATERIALS AND METHODS

Cell Culture

Two cell lines were used to investigate the effect of intracellular ice formation on membrane integrity. The first was the Madin Darby Canine Kidney (MDCK; CCL34 ATCC) epithelial cell line. These cells were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential media (MEM) supplemented with 10% v/v fetal bovine serum (all components from GIBCO Laboratories, Grand Island, NY). Cells were grown in tissue culture flasks (25 cm^2; Corning Glass Works, Corning, NY) and harvested by exposure to a 0.25% trypsin-EDTA solution (GIBCO) for 10 min at 37°C. The MDCK cells were re-suspended in supplemented MEM to obtain cell suspensions and then plated on sterilized cover slips (12 mm circle, Fisher Brand) at a concentration of 2x10^4 cells/mL. The cover slips were kept in petri dishes in an incubator for 3 d to allow the growth of a confluent monolayer.

The second cell line was the V-79W line of Chinese hamster fibroblasts. Cells were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential medium (MEM) with Hanks' salts, 16 mmol/L sodium bicarbonate, 2 mmol/L L-glutamine and 10% fetal bovine serum supplemented with antibiotics (penicillin G (50 units/mL), streptomycin (50 μg/mL)) (all components from GIBCO Laboratories, Grand Island, NY). Cells were grown in tissue culture flasks (25 cm²; Corning Glass Works) and harvested by exposure to a 0.25% trypsin solution (GIBCO) for 10 min at 37°C. The fibroblasts were resuspended in supplemented MEM. Sterilized cover slips (12 mm circle, FISHER Brand) were placed in a petri dish (FISHER Brand, 100x15 mm) and covered with 15 mL of supplemented MEM containing $3x10^5$ cells. The petri dishes were incubated for 3 days to allow the growth of a confluent monolayer.

Cryomicroscope and Video System

The cryomicroscope and video system used for this study is described in detail elsewhere (4,38). Briefly, it consisted of a Zeiss fluorescent microscope (Carl Zeiss, Germany), a CCD video camera (ZVS-47DEC, Carl Zeiss), a video recorder (GX4, Panasonic, Japan) and a convection cryostage similar to the design described by Diller *et al.* (14). The cryostage was connected to a computer-controlled interface (Great Canadian Computer Company, Spruce Grove, Canada). The computer monitored the temperature by analyzing the voltage from a thermocouple on the stage and via a proportional controller circuit, heat was added as necessary to allow the stage to follow a user defined thermal protocol.

Intracellular Ice Formation

The formation of intracellular ice was detected using the standard flash method (12,24) in conjunction with a fluorometric technique (4,23). The fluorescent nucleic acid probe SYTO 13 (Molecular Probes, Eugene, OR) was used to assist in the identification of IIF in the MDCK and V-79W confluent monolayers. The formation of intracellular ice was determined to occur if there was a distinct disruption in the pattern of fluorescent staining upon freezing. Single attached cells and confluent cell monolayers were stained with SYTO and allowed to incubate at 22°C for 10 min (MDCK) or 2 min (V-79W) prior to freezing. The incidence of IIF was calculated as the percent of cells that form intracellular ice compared to total cells in the sample.

Incidence and Kinetics of Membrane Damage

A dual fluorescent staining technique was used for the quantitative assessment of the integrity of the cell plasma membrane (Appendix A). SYTO 13, a permeant live cell nucleic acid dye (1.25 µM) and ethidium bromide (EB; Sigma Chemical Company, Mississuaga, ON; 2.5 µM) where used to differentially stain the cells. Percent survival based on membrane integrity was calculated as the number of SYTO positive cells over the total number of cells (SYTO and EB positive) using equation A-1.

The kinetics of membrane damage in V-79W and MDCK single attached cells and confluent monolayers was examined. Samples were cooled at 25°C/min to -10°C on a convection microscope and then nucleated with a cold copper probe. The samples were held at this temperature for 5 minutes and then warmed at 25°C/min to 22°C. This is the standard protocol that was used in chapter 2 to study the effects of intracellular ice formation on cell viability. From the instant of ice nucleation (t=0), membrane damage was recorded as a function of time. Membrane damage was determined as the percentage of cells scoring EB positive at time t (EB) compared to the total number of cells that were EB positive post-thaw (EB_{TOTAL}) and is denoted as EB/EB_{TOTAL}. While the term membrane damage is used, the time dependent nature of this response is likely a result of the effect of temperature on ethidium bromide diffusion and not a representation of actual membrane damage.

Overall Experimental Design

V-79W and MDCK cells were cultured to confluency on glass cover slips over 3 d at 37°C in supplemented media. The confluent monolayers were stained with 12.5 µM SYTO 13 and 25 µM ethidium bromide (EB) to assist in the detection of intracellular ice (40) and to allow for the differential assessment of membrane integrity (51). The glass cover slips were transferred to a convection cryomicroscope and cooled at 25°C/min to a predetermined experimental temperature where ice was initiated with a cold copper probe. The occurrence of intracellular ice formation was indicated by a sudden darkening of the cytoplasm

(12,24) or the disruption of the fluorescent staining (1,4,23). The samples were then warmed at 25°C/min to 22°C and the incidence of membrane damage was determined. The entire freezing procedure was videotaped for later analysis.

4.3 RESULTS

Relationship between Membrane Damage and IIF

The incidence of membrane damage and intracellular ice formation for V-79W and MDCK single attached cells and confluent cell monolayers is summarized in Tables 4.1 to 4.4. In these tables, the effects of nucleation temperature on five different conditions of the cell post-thaw are given. The first column (% Green) represents the percentage of cells that have an intact cell plasma membrane post-thaw. The second column (% Red) is the percentage of cells that have sustained damage to the plasma membrane and stain positive with ethidium bromide. The third column (% Green + II) represents the percentage of cells that formed intracellular ice and have an intact plasma membrane following thawing. The fourth column (% Red + II) depicts the percentage of cells that display a damaged plasma membrane following intracellular ice formation. In the fifth column (% II), the percentage of cells that display intracellular ice is listed. All of the values are given as a percentage of the total number of cells in the sample. The incidence of intracellular ice (% II) and membrane damage (% Red) from Tables 4.1 to 4.4 are summarized in Figures 4.1 to 4.4.

Microscopic Evaluation of Membrane Damage

In Figure 4.5 to 4.7 we see the effect of intracellular freezing on the plasma membrane integrity of a MDCK confluent monolayer cooled at 25°C/min to –9°C, frozen and then thawed at 25°C/min to 22°C. Prior to ice nucleation, all of the cells display an intact plasma membrane and a uniformly stained nucleus (Figure 4.5). After ice nucleation, and while the sample is still frozen at –9°C (Figure 4.6) all of the cells display a disruption in the fluorescent staining (honeycomb pattern), and ethidium bromide could be seen leaking into specific cells. In Figure 4.7 we see that following thawing all of the cells still display a disruption in the fluorescent staining pattern (honeycomb pattern) and some cells show damaged plasma membranes. While the incidence of IIF and membrane damage were dependent on the cell type, morphology and nucleation temperature, these general observations were consistent for both V-79W and MDCK single attached cells and confluent monolayers (data not shown).

Kinetics of Membrane Damage

The kinetics of membrane damage detection for V-79W single attached cells and confluent monolayers and MDCK single attached cells and confluent monolayers frozen using the standard cryomicroscopy protocol is shown in Figure 4.8a, 4.10a, 4.9a and 4.11a respectively. In the V-79W and MDCK confluent monolayers as well as the MDCK single attached cells, prior to the start of the thawing process (t = 300 s), the number of cells with damaged plasma membranes (EB positive) equals the total number of cells that will have damaged membranes post-thaw. In V-79W single attached cells, only 88.2 ± 11.1% of damaged cells can be detected prior to the commencement of thawing, but this will rise to 100% prior to the sample reaching 22°C. The time for 50% of the membrane damage to be observed (t_{50}) was 128.0 ± 7.1 s for V-79W single attached cells; 53.0 ± 4.4 s for MDCK single attached cells; 82.9 ± 3.9 s for V-79W confluent monolayers and 93.7 ± 5.6 s for MDCK confluent monolayers.

An additional experiment was performed to verify that membrane damage occurred during freezing and not during thawing. In this experiment V-79W and MDCK single attached cells and confluent monolayers were cooled at 25°C/min to -10°C, nucleated and held at –10°C for 15 min before being warmed at 25°C/min to 22°C. This 15 min holding period was significantly longer than the time that was required for the complete detection of membrane damage (as observed from figures 4.8a to 4.11a). The kinetics of membrane damage detection for V-79W and MDCK single attached cells and confluent cell monolayers is shown in Figures 4.8b to 4.11b. There was no statistically significant difference between the results from the two different experimental protocols. The t_{50} value was 127.5 ± 14.8 s for V-79W single attached cells; 65.6

± 2.8 s for MDCK single attached cells; 103.8 ± 10.0 s for V-79W confluent monolayers and 103.8 ± 20.4 s for MDCK confluent monolayers.

4.4 DISCUSSION

Cell-Cell Contact Affects Membrane Integrity

For V-79W and MDCK single attached cells (Tables 4.1 and 4.2), there is a direct correlation between the presence of intracellular ice and membrane damage. For both cell types the % Red and % II are identical ($p<0.005$, students' t-test) indicating a strong correlation between membrane damage and the presence of intracellular ice. It is even more informative to look at the incidence of membrane damage (% Red) compared to the incidence of cells that froze intracellularly and have a damaged membrane (% Red + II). Comparing the data from these two columns suggests that the cells that display a damaged membrane post-thaw are the same cells that formed intracellular ice. Clearly, in single attached cells, membrane damage and intracellular ice formation are intimately linked. This is consistent with the results reported for V-79W fibroblasts (4).

In tables 4.3 and 4.4, and figures 4.3 and 4.4 we see a different relationship between the presence of intracellular ice and membrane damage for V-79W and MDCK confluent monolayers. At low subzero temperatures, there is an uncoupling of membrane damage with the presence of intracellular ice. At these temperatures, there is an increased difference between the percentage of cells with damaged plasma membranes (% Red) and intracellular ice (% II). Expectedly, there is an increase in the percentage of cells with intact membranes that formed intracellular ice (% Green + II). Membrane damage remains fairly constant over a range of nucleation temperatures for both V-79W and MDCK monolayers. This unique phenomenon has previously been reported for V-79W fibroblasts (4), and is now demonstrated for MDCK cells.

Membrane Damage in Frozen Samples

The data presented in Tables 4.1 to 4.4 and Figures 4.1 to 4.4 demonstrate the correlation between IIF and membrane damage, however it does not provide any insightful information about the sequence of events surrounding the formation of intracellular ice and membrane damage. If one looks at the images that were used to collect this data, we can begin to better understand the process of intracellular ice formation. Comparing Figure 4.5 and 4.7 reveals the strong relationship between intracellular freezing and plasma membrane integrity. In the MDCK monolayer all of the cells that have a disrupted plasma membrane also form intracellular ice. There were not any observed cases where membrane damage was evident and IIF did not occur. This was consistent for V-79W and MDCK single attached cells and confluent monolayers. However, for MDCK and V-79W confluent monolayers, it was common to observe intracellular ice formation and no membrane damage. This is in agreement with the observations of Acker and McGann (4).

The surprising observation was that ethidium bromide could be seen leaking into certain cells in the confluent monolayer while the sample was still frozen (Figure 4.6). After ice nucleation, and while the sample was still frozen at –9°C, we observed that all of the cells displayed a disruption in the fluorescent staining pattern (honeycomb pattern). This has been directly correlated with the formation of intracellular ice (1,4). Following the formation of intracellular ice, sections of the cell began to change color from bright green to orange, indicating that ethidium bromide was leaking into the cell. For ethidium bromide to fluoresce red it must bind to nucleic acids and, hence must be able to cross the plasma membrane. This is a very important observation, as it provides direct, concrete evidence that membrane damage occurred during freezing.

Membrane Damage Occurs During Freezing and Not Thawing

Using fluorescent cryomicroscopy, we are able to carefully deduce when damage occurs during freezing and thawing. In Figures 4.8a to 4.11a, the kinetics of membrane damage for V-79W and MDCK single attached cells and confluent monolayers are shown. From these figures, it would appear that membrane damage occurs early during the nucleation and freezing event allowing sufficient time for the diffusion of ethidium bromide into the cell. As there was no significant increase in the percentage of cells suffering damage to the plasma membrane during the thawing process or following the return to room temperature, all of the damage done to the cells must have occurred during freezing. An additional test was conducted to confirm that the rise in membrane damage observed in Figures 4.8a to 4.11a was not influenced by the freezing protocol. The results from this experiment indicate that there was no effect of increasing the holding time from 5 to 15 min on the kinetics of membrane damage detection. This provides additional confirmation that membrane damage occurs during freezing and is completed prior to the start of thawing.

While there are some numerical differences between the t_{50} values reported for the different cell types and cell morphologies, the author does not believe that these are significant. Due to the nature of the experimental protocol, the accuracy of data between 0 and 100% membrane damage is suspect. The measurement of the diffusion of ethidium bromide was conducted while the samples were still frozen and was masked by the presence of extracellular ice. In many cases it was difficult to determine the exact time that the cell began to display membrane damage. For this reason, the t_{50} values likely do not have any physical or practical meaning. A more accurate method of comparing the different cell types and cell morphologies would be to look at the time it took to reach 100% membrane damage. With the exception of V-79W single attached cells, complete membrane damage occurred between 200-250 s following nucleation. And in all of the cell types, there was complete detection of membrane damage before thawing was complete (375 s for 5 min hold).

The observation and measurement of ethdium bromide diffusion into a cell while it remains frozen is an excellent indication that damage has occurred during the freezing process. To date there has been no other reports documenting this behaviour. While other researchers have coupled fluorescent staining with cryomicroscpy (36,47) they did not report observing damage prior to thawing. This is likely a result of the fact that they only used a single fluorescent probe, fluorescein diacetate (FDA), which has been shown not to be a good indicator of membrane integrity (35). Also, in most cases, membrane damage was only assessed post-thaw eliminating any possibility of observing any leakage of FDA out of the cell that could have occurred while the samples were still frozen.

Membrane Damage in Confluent Monolayers

It has been previously demonstrated that a majority of the cells in confluent monolayers that form intracellular ice at low temperatures do not display a damaged plasma membrane following thawing (1,4, Table 4.3 and 4.4). It has further been shown that intracellular ice can propagate between adjacent cells in a monolayer (1,3,5,6,9,10,11,20,22,37,45,48,49,50) and has been suggested that this occurs without disruption of the plasma membrane (1,4). In an attempt to rationalize these two observations, Acker proposed that IIF occurs in a single cell resulting in the disruption of the plasma membrane. From this single cell, ice then propagates to adjacent cells via a mechanism that prevents damage to the membrane (1). If this is this case, then one should be able to observe this occurring in confluent monolayers. Using fluorescent staining techniques in conjunction with the cryomicroscope, we can explore the relationship between intracellular ice formation and membrane damage in confluent monolayers in much more detail than was previously possible.

Using the video segments collected from the cryomicroscopy of V-79W confluent monolayers, we performed a retrospective study to look at the sequence of events involved in the formation of intracellular ice and the disruption of the plasma membrane. By performing frame-by-frame analysis we determined the time that each cell underwent intracellular freezing and hence the sequence of IIF in the confluent monolayer. This was coupled to our studies on the kinetics of ethidium bromide leakage to give us the complete temporal and spatial sequence of intracellular ice formation and membrane damage. Figure 4.12 is a characteristic graph that can be generated from this data. The X/Y coordinates of the center of each cell in a sample are plotted as an open circle. All of the cells that form intracellular ice are shown as a solid circle. On top of this static data, we have highlighted the cells that form intracellular ice without any already frozen adjacent cells (open diamond). We term these independent sites of intracellular ice formation. Of these independent sites of IIF we denote those that have damaged plasma membranes (open square), and will refer to these as independent EB sites. Representing the data in this manner allows us to look closely at the relationship between intracellular ice formation and membrane damage.

In Figure 4.12 we have the results of a V-79W confluent monolayer nucleated at –7°C and then thawed. It is clear from this graph that there is a strong correlation between the cells that initially form intracellular ice and a disruption in the cell plasma membrane. In this sample 14 of the 64 (21.8%) cells that formed intracellular ice do so independent of a frozen neighbour. Of these 14 cells, 8 (57.1%) demonstrate post-thaw damage to the plasma membrane. It is unclear why the remaining 6 cells do not display a damaged plasma membrane. It is possible that following intracellular ice formation, the membrane was resealed preventing the diffusion of ethidium bromide. There is evidence in the literature to suggest that this can occur (13,21,33,38). However, a more practical explanation is that these cells are likely not true independent sites of IIF. In each case, the cells that do not display membrane damage are located in relative proximity to the edge of the field of view. It is likely that the cells were nucleated by a cell out of view and only appear to be independent as a result of the absence of a visible neighbour. The data shown in Figure 4.12 is consistent with the results obtained from other experimental samples and other nucleation temperatures.

The temporal and spatial analysis of V-79W confluent monolayers was performed for multiple temperatures of nucleation and the results are summarized in Figure 4.12. A plot of the percent independent EB sites as a function of the percent independent IIF sites indicates that there is a very strong relationship (R^2=0.9386) between the initial formation of intracellular ice in a confluent monolayer and membrane damage. We can conclude with some degree of certainty that the initial cells that form intracellular ice in confluent monolayers will have damaged plasma membranes. This provides strong evidence to support Acker's belief that ice enters random cells resulting in damage to the membranes and then ice propagates to adjacent cells (1).

Absent from these plots were the cells that developed a damaged plasma membrane following the intracellular nucleation from an adjacent cell. These 'non-independent' EB sites make up a significant percentage of the total cells in the sample that display membrane damage. In Figure 4.13, we see the same sample that is represented by Figure 4.12 with the 'non-independent' EB sites added. A proposed mechanism of damage for these cells will be presented in chapter 6.

This method of analysis could not be performed on MDCK confluent monolayers as a result of the different mechanism by which intracellular ice forms and propagates. Under most conditions, MDCK cells display complete formation of intracellular ice in all of the constituent cells (Table 4.4). It has also been shown the intracellular ice propagates in a wave-like manner through the sample (1,2). As a result, there are no truly independent IIF sites, therefore negating any benefit of performing frame-by-frame analysis of the freezing process. This will be discussed further in chapter 6.

4.5 SUMMARY

Up until now, all of the current data examining the relationship between membrane damage and intracellular ice formation and the sequence of these two events during freezing has been highly subjective and based on indirect measurements. Using a common cryomicroscope and readily available nucleic acid stains, we have developed a technique that can be used to answer one of the most elusive questions in cryobiology: does intracellular ice damage occur during freezing or during thawing? By directly observing and measuring the diffusion of a membrane impermeable fluorescent stain, ethidium bromide, into cells and correlating the results with the incidence of IIF, we have provided new evidence on the time course of IIF-related membrane damage. This study presents strong evidence that membrane damage occurs during freezing and is directly associated with intracellular ice formation.

In addition to investigating the time course of IIF and membrane damage in single attached cells, this study has provided important information on the sequence of events involved in the formation of intracellular ice in confluent monolayers. In monolayers, cells that nucleated independent of frozen neighbours have a higher probability of having a disrupted cell membrane than cells that are nucleated by cell-cell propagation.

While we can confidently link membrane damage to the freezing event, we can not conclude whether intracellular ice formation is the cause or result of the observed damage to the cell plasma membrane. In this study, we could not resolve the time difference between IIF and membrane rupture because of the temperature-dependent nature of the EB diffusion. While we could observe the instant intracellular ice forms in a cell, we could not determine if damage occurred prior to this event. As diffusion is concentration dependent, it may not be possible to determine whether IIF is the cause or result of membrane damage using the technique presented herein. However efforts should be made to couple fluorescent microscopy with high-speed videography to further investigation whether the plasma membrane is damaged before or after intracellular ice formation.

We have demonstrated that injury to cells from intracellular ice formation occurs during freezing, and that the result is damage to the plasma membrane. In confluent monolayers, the initial formation of intracellular ice corresponds with the disruption of the plasma membrane. Determining the sequential events of intracellular ice formation and membrane damage will have important implications in the refinement of the models used to describe the formation of intracellular ice in cells and tissue.

	Mean ± SEM (n=6)				
Temp. (°C)	**% Green**	**% Red**	**% Green + II**	**% Red + II**	**% II**
-6	93.4 ± 4.2	6.6 ± 4.2	1.0 ± 1.0	6.6 ± 4.2	7.6 ± 4.0
-8	48.6 ± 8.6	51.4 ± 8.6	2.9 ± 1.3	48.9 ± 8.5	51.7 ± 8.0
-10	5.3 ± 2.3	94.7 ± 2.3	3.0 ± 2.1	93.8 ± 2.9	96.7 ± 2.8
-12	0	100.0	0	100.0	100.0

Table 4.1 – Intracellular Ice and Membrane Damage - **V-79W Single Attached Cells**

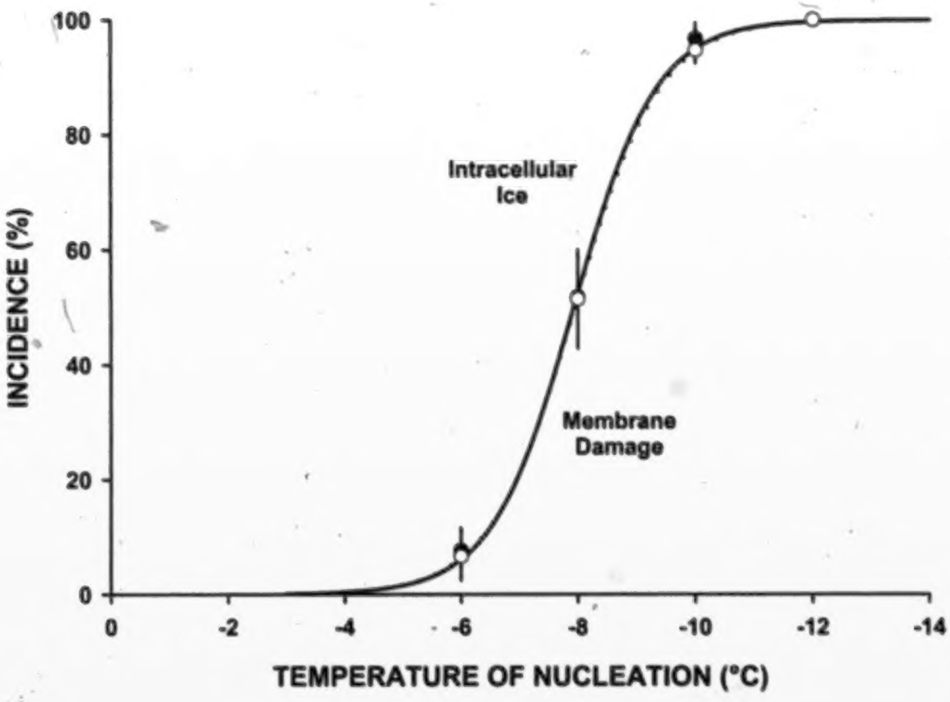

Figure 4.1 – Incidence of intracellular ice formation and membrane integrity as a function of nucleation temperature for **V-79W single attached cells**. Mean ± SEM (n=6).

	Mean ± SEM (n=6)				
Temp. (°C)	% Green	% Red	% Green + II	% Red + II	% II
-2	91.4 ± 5.4	8.6 ± 5.4	0.0 ± 0.0	8.6 ± 5.4	8.6 ± 5.4
-4	80.5 ± 5.0	19.5 ± 5.0	1.1 ± 1.1	19.5 ± 5.0	20.6 ± 5.6
-6	39.8 ± 8.0	60.2 ± 8.0	0.9 ± 0.9	60.2 ± 8.0	61.1 ± 8.3
-8	19.2 ± 3.7	80.8 ± 3.5	3.6 ± 1.2	80.0 ± 3.5	83.6 ± 3.4

Table 4.2 – Intracellular Ice and Membrane Damage - **MDCK Single Attached Cells**

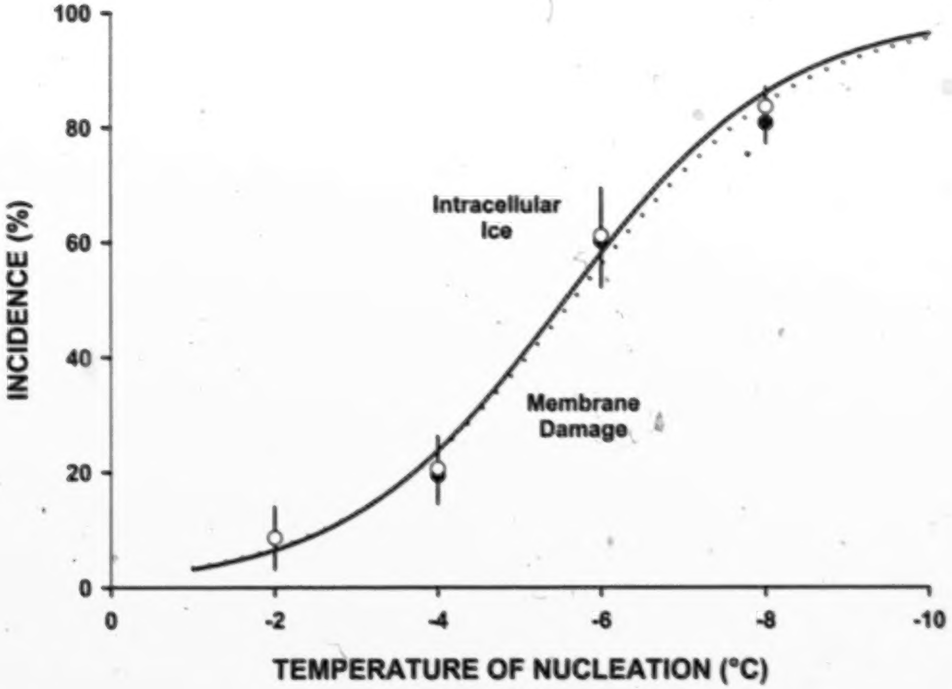

Figure 4.2 – Incidence of intracellular ice formation and membrane integrity as a function of nucleation temperature for **MDCK single attached cells**. Mean ± SEM (n=6).

	Mean ± SEM (n=6)				
Temp. (°C)	% Green	% Red	% Green + II	% Red + II	% II
-5	92.2 ± 1.6	7.8 ± 1.6	1.8 ± 0.6	5.8 ± 0.8	7.6 ± 0.7
-7	85.5 ± 1.0	14.5 ± 1.0	31.1 ± 1.7	9.5 ± 3.3	40.6 ± 1.6
-9	76.3 ± 8.4	23.7 ± 8.4	65.2 ± 9.3	22.3 ± 7.9	87.5 ± 2.4
-11	82.8 ± 5.1	17.2 ± 5.1	82.8 ± 5.1	16.8 ± 5.1	99.6 ± 0.2

Table 4.3 – Intracellular Ice and Membrane Damage - **V-79W Confluent Monolayers**

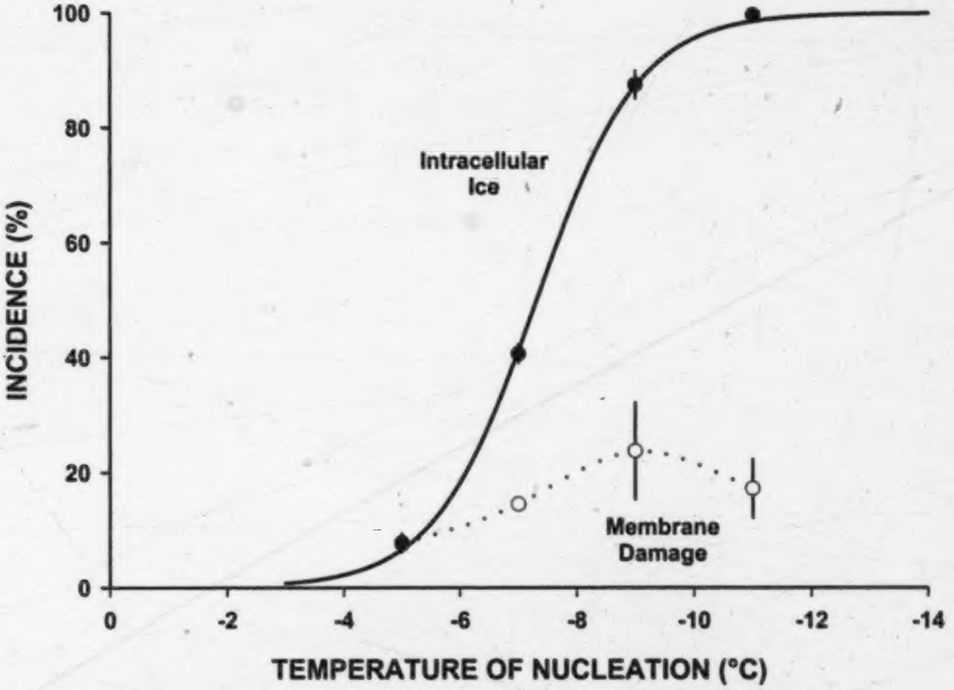

Figure 4.3 – Incidence of intracellular ice formation and membrane integrity as a function of nucleation temperature for **V-79W confluent monolayers**. Mean ± SEM (n=6).

	Mean ± SEM (n=6)				
Temp. (°C)	**% Green**	**% Red**	**% Green + II**	**% Red + II**	**% II**
-5	83.0 ± 1.8	17.1 ± 1.8	83.0 ± 1.8	17.1 ± 1.8	100.0
-7	71.6 ± 3.5	28.4 ± 3.5	71.6 ± 3.5	28.4 ± 3.5	100.0
-9	71.8 ± 4.4	28.2 ± 4.4	71.8 ± 4.4	28.2 ± 4.4	100.0
-11	71.4 ± 3.9	28.6 ± 3.9	71.4 ± 3.9	28.6 ± 3.9	100.0

Table 4.4 – Intracellular Ice and Membrane Damage - **MDCK Confluent Monolayers**

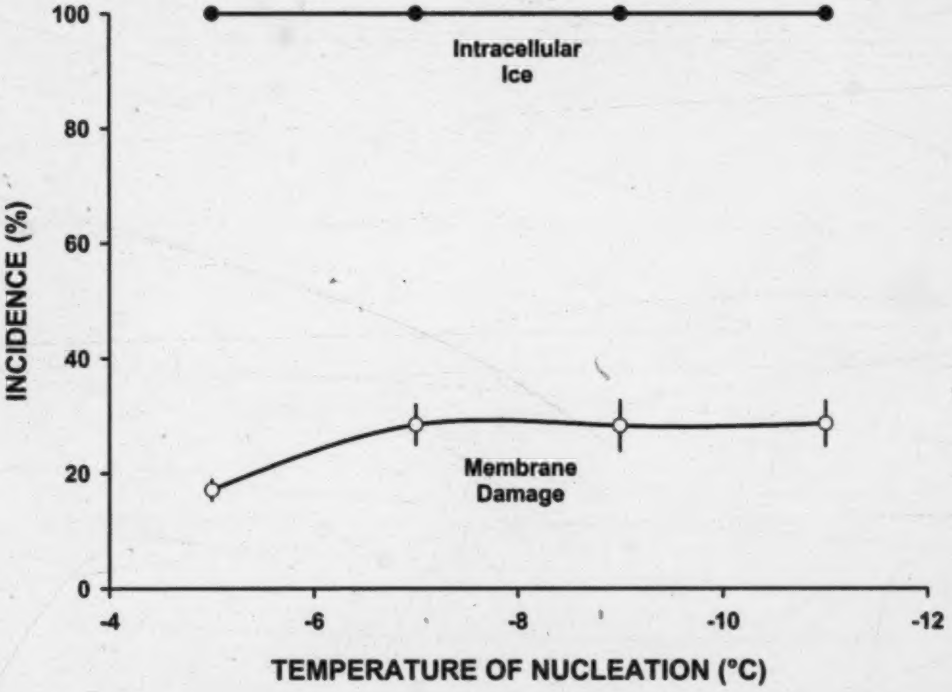

Figure 4.4 – Incidence of intracellular ice formation and membrane integrity as a function of nucleation temperature for **MDCK confluent monolayers**. Mean ± SEM (n=6).

Figure 4.5 – Effect of intracellular ice formation on membrane integrity - prefreeze. A MDCK confluent monolayer was stained with SYTO / EB and cooled at 25°C/min to -9°C on a convection cryomicroscope. Prior to ice nucleation, all of the cells display an intact plasma membrane and a uniformly stained nuclei.

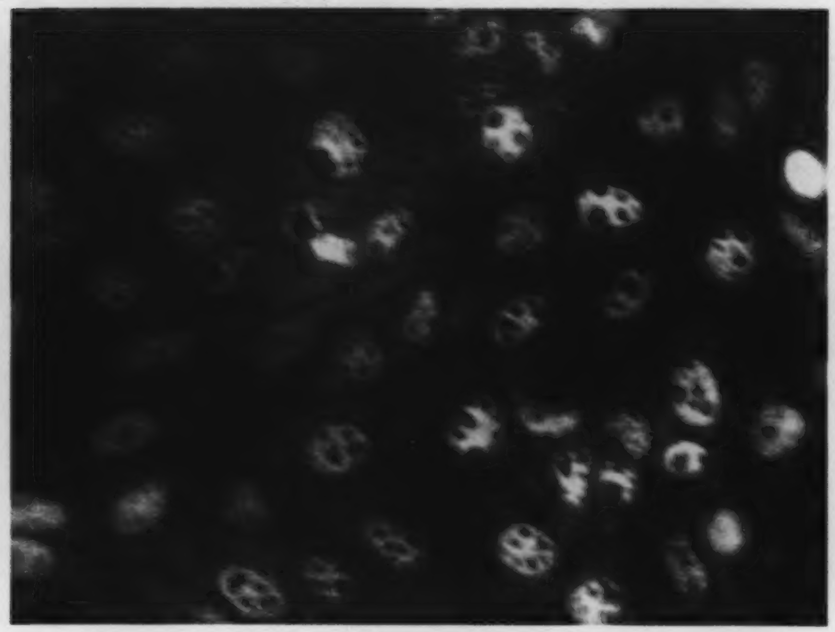

Figure 4.6 – Effect of intracellular ice formation on membrane integrity - frozen. A MDCK confluent monolayer was stained with SYTO / EB and cooled at 25°C/min to -9°C on a convection cryomicroscope. After ice nucleation, all of the cells display a disruption in the fluorescent staining pattern (honeycomb pattern) and in some cells ethidium bromide can be seen leaking into the cells.

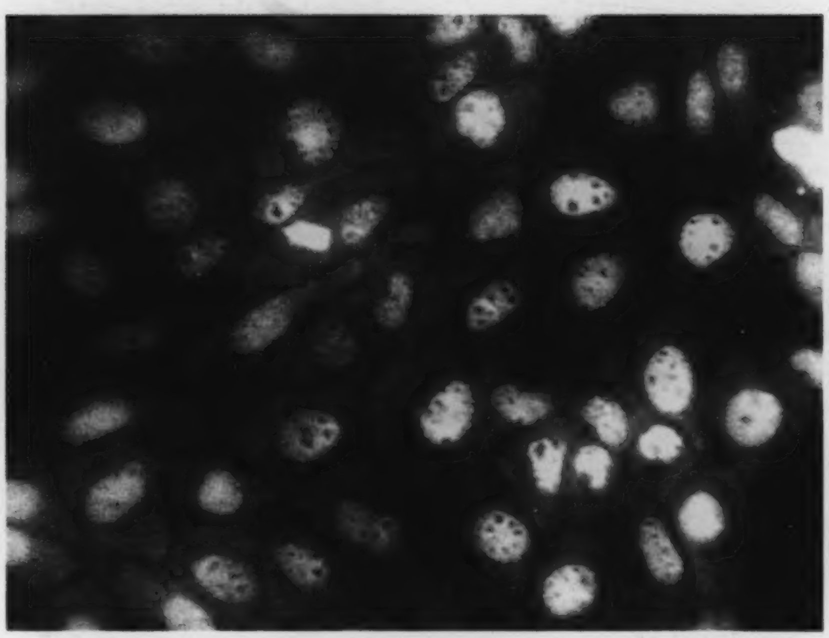

Figure 4.7 – Effect of intracellular ice formation on membrane integrity - post-thaw. A MDCK confluent monolayer was stained with SYTO / EB and cooled at 25°C/min to -9°C on a convection cryomicroscope. After ice nucleation and thawing (25°C/min to 22°C) all of the cells still display a disruption in the fluorescent staining pattern (honeycomb pattern) and some cells shown a damaged plasma membranes

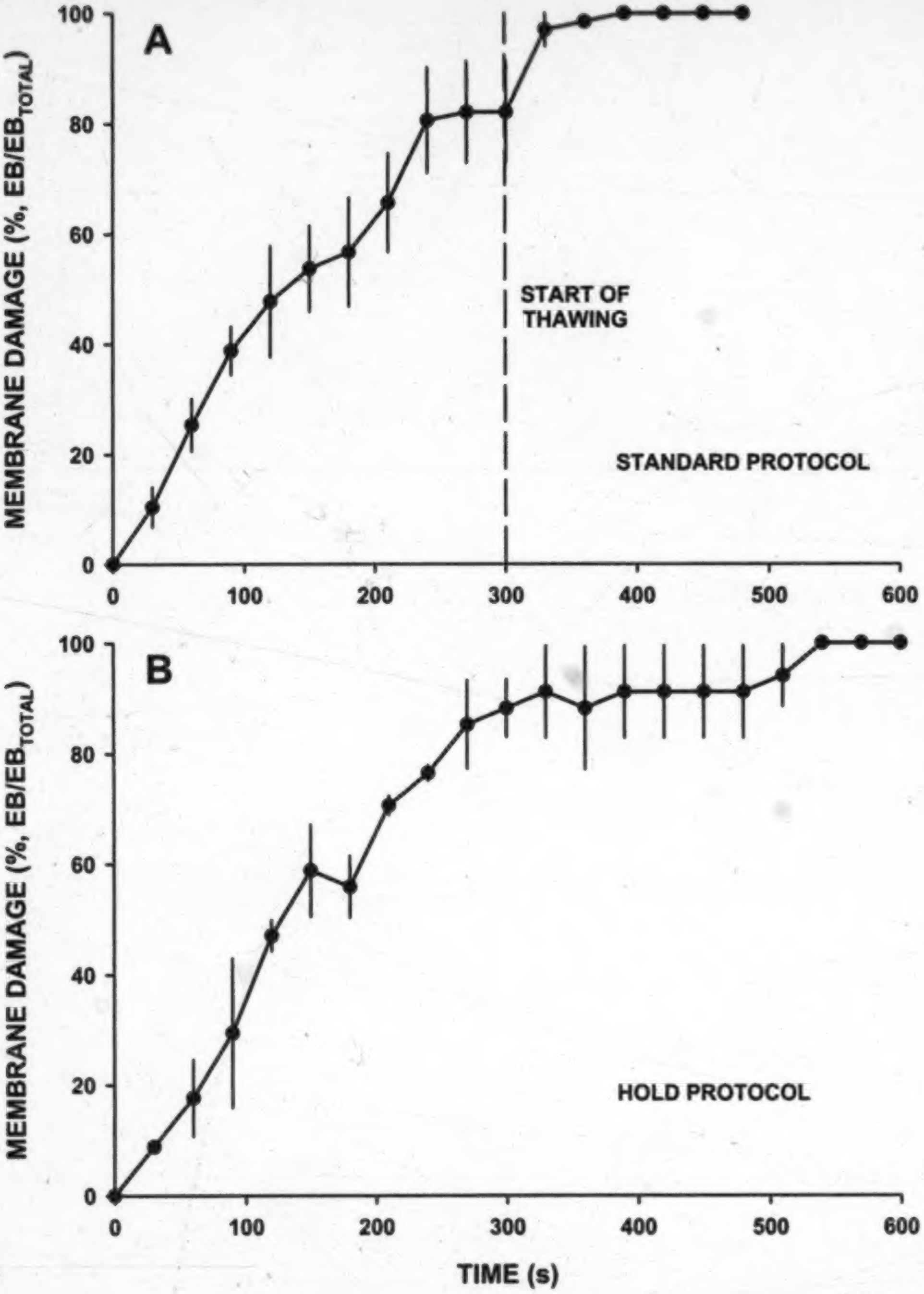

Figure 4.8 – Kinetics of membrane damage detection for **V-79W single attached cells**. Data was collected during a standard cryomicroscopy protocol (A) or a modified protocol where the sample was held at -10°C for 15 min (B). Mean ± SEM (n=6).

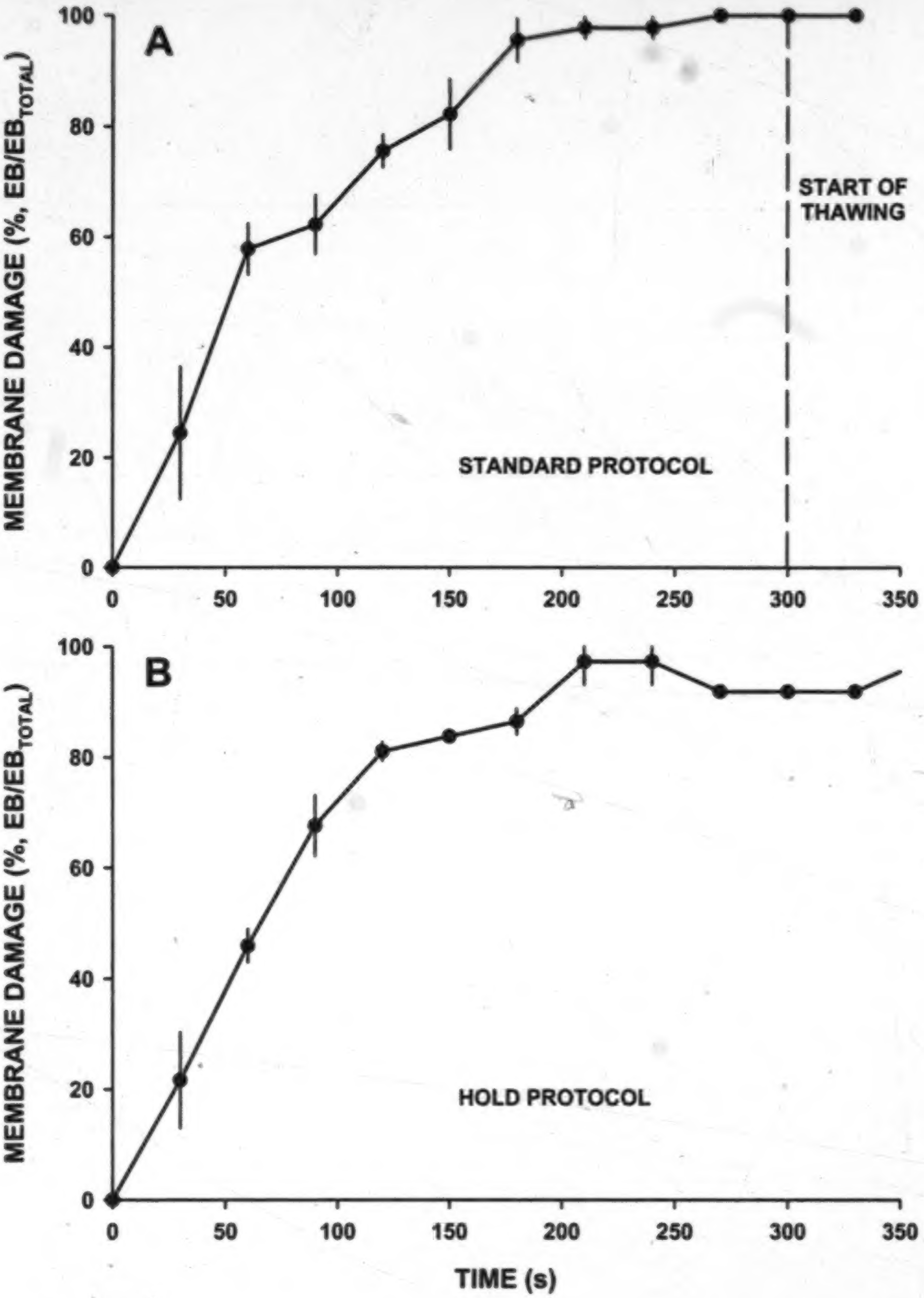

Figure 4.9 – Kinetics of membrane damage detection for **MDCK single attached cells**. Data was collected during a standard cryomicroscopy protocol (A) or a modified protocol where the sample was held at -10°C for 15 min (B). Mean ± SEM (n=6).

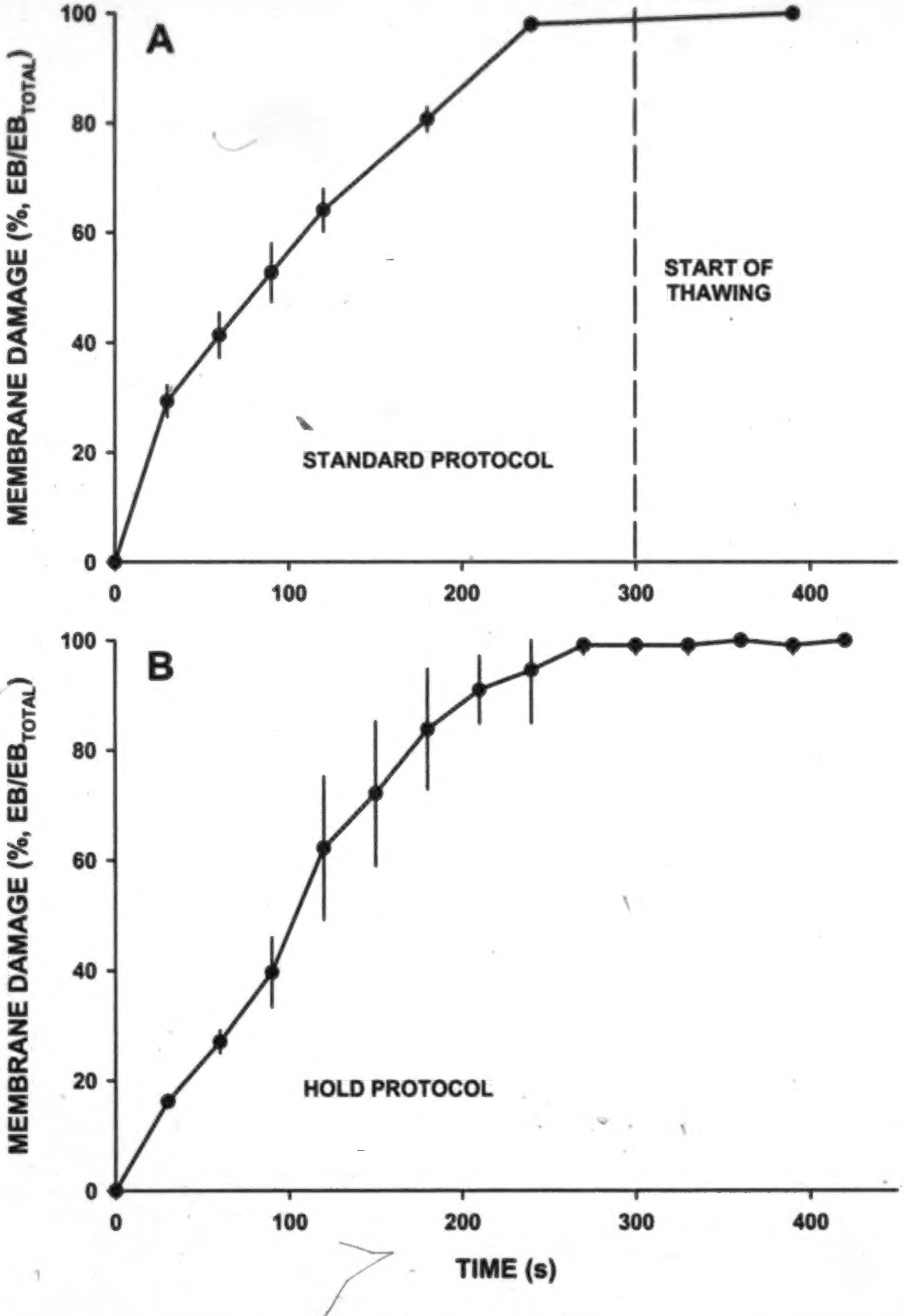

Figure 4.10 – Kinetics of membrane damage detection for **V-79W monolayers.** Data was collected during a standard cryomicroscopy protocol (A) or a modified protocol where the sample was held at -10°C for 15 min (B). Mean ± SEM (n=6).

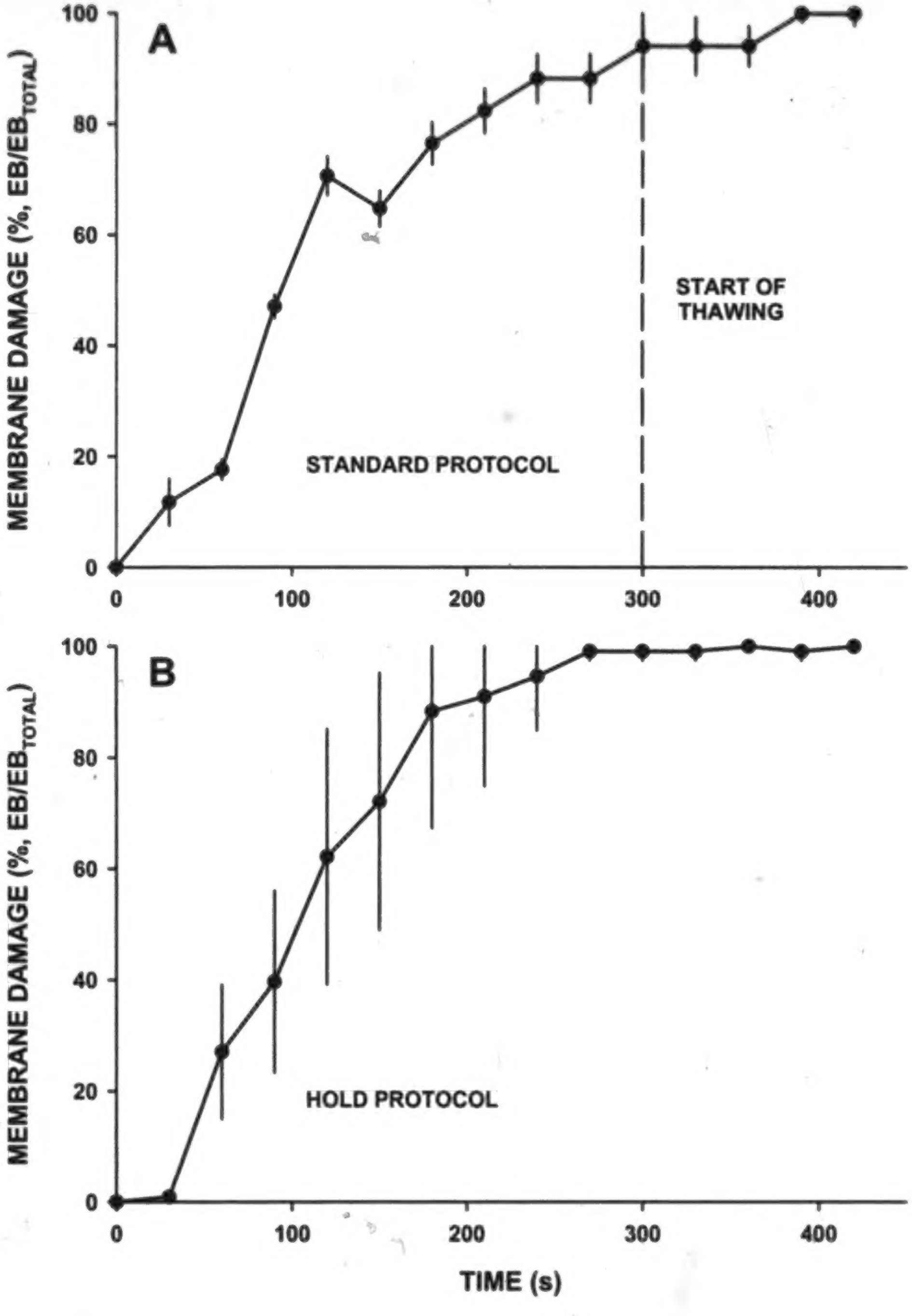

Figure 4.11 – Kinetics of membrane damage detection for **MDCK monolayers**. Data was collected during a standard cryomicroscopy protocol (A) or a modified protocol where the sample was held at -10°C for 15 min (B). Mean ± SEM (n=6).

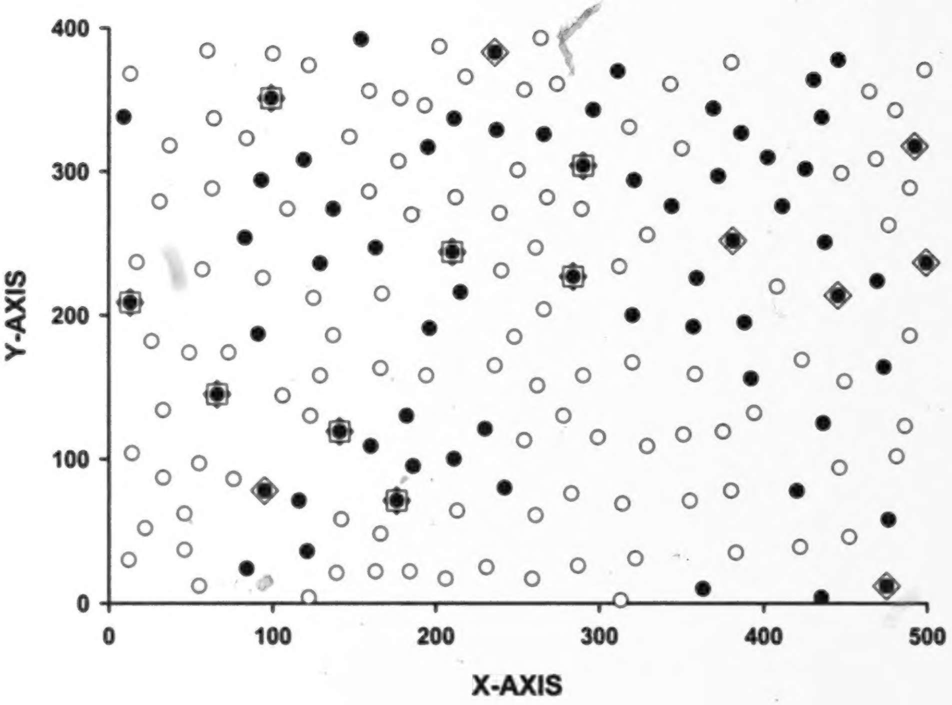

Figure 4.12 – Spacial and temporal analysis of IIF and membrane disruption in a V-79W confluent monolayer nucleated at -7°C. The X/Y coordinates of the center of each cell in the sample are plotted as an open circle. All of the cells that form intracellular ice are shown as a solid circle. On top of this static data, is highlighted the cells that form intracellular ice without any already frozen adjacent cells (open diamond). Of these independent sites of IIF we can denote those that have a damaged plasma membrane (open square).

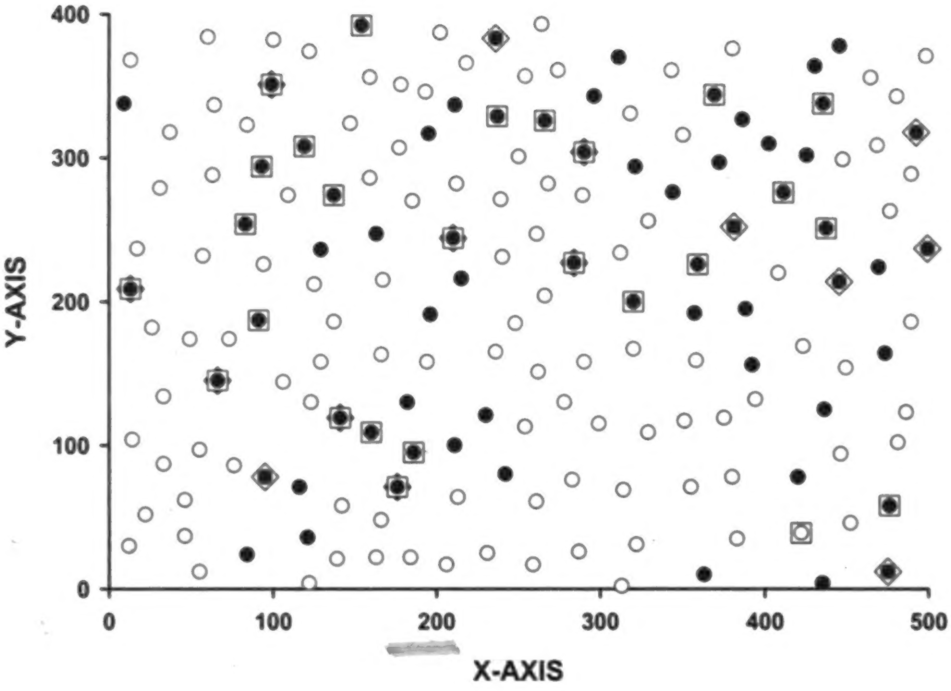

Figure 4.13 – Spacial and temporal analysis of IIF and membrane disruption in a V-79W confluent monolayer nucleated at -7°C. The X/Y coordinates of the center of each cell in the sample are plotted as an open circle. All of the cells that form intracellular ice are shown as a solid circle. On top of this static data, is highlighted the cells that form intracellular ice without any already frozen adjacent cells (open diamond). **All of the cells with damaged plasma membrane are shown** (open square).

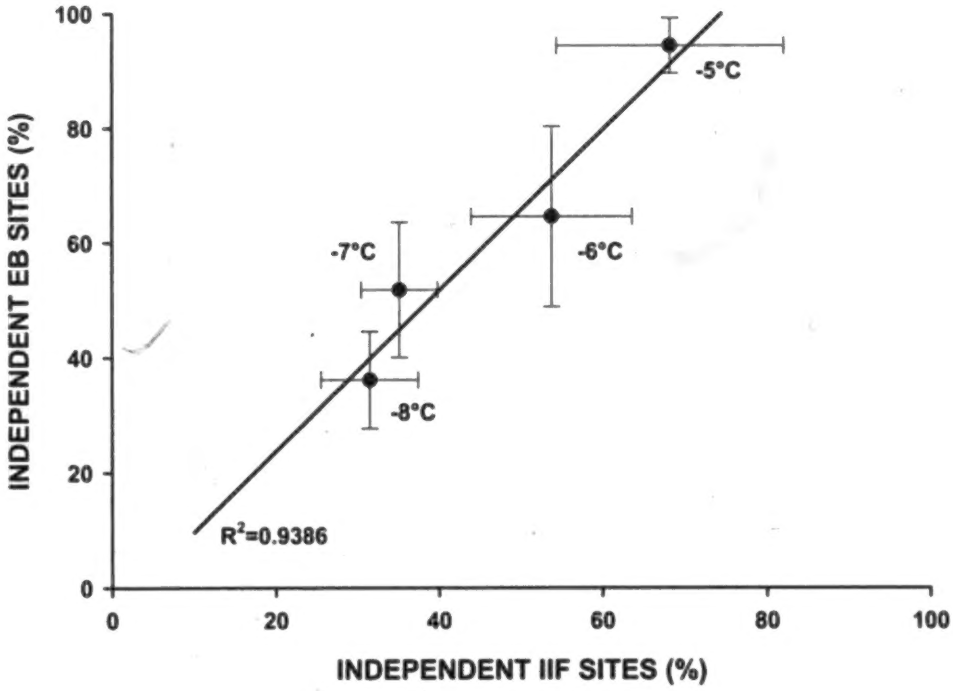

Figure 4.14 – Correlation between sequence of membrane damage and IIF in V-79W confluent monolayers. A plot of the percent independent EB sites as a function of the percent independent IIF sites. Mean ± SEM (n=4).

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CHAPTER FIVE
CELL-CELL PROPAGATION VIA GAP JUNCTIONS

5.1 INTRODUCTION

Tissues are a complex arrangement of multiple cells and cell types which interact together to perform a function. During freezing, cell-cell and cell-matrix connections have been shown to significantly affect the response of tissues to freezing and thawing. One of the best-documented effects of cell-cell coupling has been the propagation of intracellular ice that occurs between adjacent cells in confluent monolayers and tissue systems. Individuals investigating the freezing of plant tissue (6,9,10,11,18,20,24,25,27,30) first documented this phenomenon. In fact, some of the first observations of freezing using a cryomicroscope noted the relationship between cell contact and intracellular ice (11,25,27). However, observations of induction of intracellular ice between adjacent cells have not been limited to studies with plant tissue. There have been reports of cell-cell propagation of intracellular ice in muscle fibres (11), salivary glands (7), cultured cells (3,5,17) and even intact Antarctic nematodes (33). In most cases, the observation of cell-cell propagation of intracellular ice was made secondary to the study being conducted. Only recently has there been an interest in the role that cell-cell and cell-matrix interactions play in the freezing response of tissue systems (1,4,16).

To validate the concept of cell-cell propagation of intracellular ice, one needs to ensure that two conditions can be meet. The first is to demonstrate that the formation of ice in the constituent cells is in fact a result of propagation and not a result of the spontaneous development of internal freezing in adjacent cells. The second requirement is that there be a plausible means by which intracellular ice can propagate between cells. If both of these requirements can be shown to be present in tissue systems, then it is conceivable that cell-cell propagation may occur.

There have been a number of studies that have demonstrated the non-random nature of IIF in tissue systems (9,10,27,29,30). In a series of experiments between 1974 and 1980, Brown demonstrated that ice formation in the constituent cells of cucumber (10) and onion tissue (9) was influenced by the presence of ice in an adjacent cell. By mapping the sequence of IIF, he was able to conclude that there were two types of cellular nucleation: a random process without interference from adjacent cells and another in which adjacent cells freeze sequentially. These results were confirmed in 1998 by Tsuruta *et al.* who used high-speed video microscopy to examine the propagation of intracellular ice in onion epidermal tissue (29). Tsuruta *et al.* provide convincing optical verification of the non-random nature of cellular nucleation. Finally, using statistical analysis, Acker and McGann have demonstrated that the probability of intracellular ice formation increases when an adjacent cell is already frozen (3). By qualitatively assessing the degree of randomness of IIF in fibroblast confluent monolayers they demonstrated that cell-cell contact could facilitate the induction of intracellular ice between adjacent cells. From these studies, it is proposed that the formation of intracellular ice in tissue systems occurs in a well-defined, non-random nature.

Since intracellular ice has been shown to propagate between adjacent cells, the logical extension would be to determine how this occurs. There are two current theories as to the mechanism by which intracellular ice can propagated between cells. Tsuruta *et al.* (29) have suggested that cell-cell propagation of intracellular ice occurs via surface-catalyzed nucleation (SCN). The SCN model for intracellular ice formation was proposed by Toner *et al.* in 1990 and is based on the premise that the cell plasma membrane, interacting with extracellular ice, is able to act as an efficient nucleation site (28). While the SCN theory was developed to explain intracellular nucleation, it does not preclude the possibility that intracellular ice functions in a similar manner as extracellular ice to cause intercellular nucleation. Through carefully controlled experiments, Tsuruta *et al.* were able to observe the induction of ice from one cell to another and correlate this behavior with the SCN theory (29,30). Surface catalyzed nucleation has also been suggested to be a mechanism by which ice can propagate between adjacent fibroblasts in a confluent monolayer (1,3). Acker has proposed that internal ice may alter the structure of the membrane allowing for the external surface of the adjacent cell membrane to become an efficient ice nucleating site (1). The induction of ice between adjacent cells might therefore occur as a result of the ice in one cell, using the adhesion between cells, to nucleate ice in adjacent cells. However, recent studies that have applied the SCN theory to the modeling of IIF in tissues systems have resulted in less than ideal results due to the fact that the original SCN theory does not consider propagative cell freezing (30).

An alternative mechanism for the propagation of intracellular ice between adjacent cells was proposed by Berger and Uhrik (7). They proposed that intracellular ice can propagate between cells via gap junctions. Gap junctions are intercellular junctions that permit the passage of small molecules between cells. Using single strands of salivary tissue, Berger and Uhrik demonstrated the induction of ice between cells and the inhibition of this propagation by the addition of chemical agents that uncoupled adjacent cells (7). In 1997, Acker and McGann compared the rate, pattern and cumulative incidence of intracellular ice formation in confluent monolayers of cultured cells that did and did not have gap junctions (1,2). They concluded that gap junctions likely influenced the induction of intracellular ice between adjacent cells. Critical to all of this work has been the assumption that ice can grow through small-diameter pores.

The concept of ice growth through a pore has been central to a long-standing theory in cryobiology. In 1960 Mazur proposed that intracellular ice formation can occurs as external ice passes through pores in the plasma membrane (Figure 5.1; 21). He followed this up in 1965 with a detailed mathematical description of this phenomenon (22,23). Mazur used the following equation, which he considered to be analogous to the Kelvin equation, to model the change in equilibrium ice crystal radius with temperature:

$$\Delta T = \frac{2v_1 T_f \sigma_{SL} \cos\theta}{aL_f} \qquad (5\text{-}1)$$

where ΔT is the difference in freezing point of a planar ice-water interface (T_f) and the freezing point of water in the pore of radius a; σ_{SL} is the interfacial tension between the ice and the liquid water; v_1 is the molar volume of pure water; θ is the contact angle between the ice-water interface and the pore wall; and L_f is the molar heat of fusion (22,23). Using this model, Mazur suggested that the equilibrium radius of an ice crystal below -10°C was sufficient to pass through pores of 8 Å if the contact angle of the ice-water interface with the pore wall was approximately 75°. However, in his application of the equation, Mazur was required to make a critical assumption that would later affect his ability to validate the theory. As the contact angle between ice, water and the pore was unknown, Mazur assumed it to be 0°. This was required to calculate the pore diameter (ice crystal diameter) that agreed with the observed temperature where extracellular ice propagation was prevented by the membrane. A 0° contact angle would require a pore diameter of 30 Å for ice to propagate at –10°. Mazur provided evidence to suggest that the angle was closer to 80° as a result of the unique properties of ice in transmembrane pores; however, without further evidence of the temperature-dependence of the ice propagation through pores of a known diameter, the pore theory could not be validated.

Until recently, it was not possible to prove or disprove Mazur's pore theory as a result of a lack of accurate data on the physical interaction of ice and pores. However, the work by Berger and Uhrik on the propagation of ice via gap junctions provides very strong evidence to support the pore theory (7). In their experimentation on salivary gland tissue, they observed a strong temperature dependence on the propagation of ice between adjacent cells. As the temperature increased, there was a defined point at which intercellular ice induction was not observed. However due to the nature of the experiment, the exact temperature at which intracellular ice propagation ceased could not be determined. This strongly supports the idea that there is a critical radius, and hence a temperature at which ice can not propagate between adjacent cells. Accurately determining this temperature and correlating it with known physical properties of gap junctions would permit one to validate the assumptions made by Mazur using his equation and hence Mazur's pore theory.

We will demonstrate that the temperature-dependence of ice propagation in confluent monolayers agrees well with Mazur's pore theory providing strong evidence for the induction of intracellular ice via gap junctions. This will satisfy the requirement for a feasible method for ice induction between adjacent cells, and will corroborate the observed propagation of intracellular ice. If gap junctions facilitate the propagation of ice from one cell to another, then this would be an important element in the development of a model to describe the formation of intracellular ice in tissues.

5.2 THEORETICAL DERIVATION

In a more careful thermodynamic analysis, Elliott derived the following equation (13):

$$\Delta T = \frac{2\sigma_{SL} v_2 T^{o}_{fp} \cos\theta}{aL_f} + \frac{v_3 \pi^L T^{o}_{fp}}{L_f} \qquad (5\text{-}2)$$

where ΔT is the difference in freezing point of a planar ice-water interface and the freezing point of water in the pore of radius a; σ_{SL} is the interfacial tension between the ice and the liquid water; v_2 is the molar volume of ice; v_3 is the molar volume of the solution; θ is the contact angle between the ice-water interface and the pore wall; π^L is the osmotic pressure of the solution; L_f is the molar heat of fusion; and T^{o}_{fp} is the freezing point of pure water.

The equation derived by Mazur (5-1), which is related to the Kelvin equation, is incomplete and only an approximation for the effect of temperature on the radius of an ice crystal. In his derivation, Mazur does not consider the effect that solutes have, and assumes that there is pure water on both sides of the pore. Clearly this is not the condition that exists in biological systems. The result is that Mazur neglects to consider the effect that solutes have on the freezing point of water in a pore which is represented by the second term of the right hand side of Equation 5-2. An additional error made by Mazur resulted in the appearance of the molar volume of liquid water in Equation 5-1 rather than the molar volume of ice as appears in Equation 5-2. Equation 5-2 corrects this error. In more general terms, Mazur's derivation is not thermodynamically correct (12), as it can not satisfy the requirement that the chemical potential of the ice is equal to the chemical potential of the liquid and the Laplace equation simultaneously. At equilibrium these two equations must both be satisfied (12).

For applications of liquid-solid equilibria it is critical that the correct equation be used (12). In this case, the error associated with using the equation derived by Mazur, and the equation derived by Elliott is approximately 10% (Figure 5.2) even without considering the solute effects.

5.3 APPLICATION OF ELLIOTT'S EQUATION

Consider the propagation of ice through a pore that connects two cells. In this situation we have intracellular ice on one side of the pore, and supercooled cytoplasm on the other. We wish to determine the minimum temperature across the pore that will permit the propagation of an ice crystal. Stated differently, what is the temperature that will correspond with an ice crystal of suitable size to pass through the pore. To answer this we will use the Elliott equation (Equation 5-2).

Of the parameters in Equation 5-2, only the interfacial tension (σ_{SL}) and the contact angle (θ) are unknown. From the work of Fletcher (14), Mazur proposed that the interfacial tension for ice-pure water interfaces is in the range of 1.7×10^{-2} J/m² at –40°C to 2.0×10^{-2} J/m² at 0°C (22,23). We will assume that the interfacial tension is linear with temperature. The only other unknown is the contact angle of the ice-water interface. In his work, Mazur concluded that the contact angle is much greater than 0° and most likely approaches 90° as a result of the unique structure of water in transmembrane pores (22,23). Recent evidence on the interaction and structure of water in transmembrane pores (8,32) would support this assumption.

With these parameters we are able to model the behavior of intracellular ice propagation via gap junctions. Using Equation 5-2 and data from Appendix B, we can plot the capillary freezing point depression as a function of pore radius for various contact angles (Figure 5.3). For a given pore radius, as the contact angle increases, the capillary freezing point depression decreases and approaches the freezing point of the solution at 90°. Similarly, as the pore radius increases, the freezing point will decrease for any given contact angle. This means that the larger the pore, or the greater the contact angle, the lower the degree of supercooling that is required across the pore for intercellular ice propagation to occur.

A critical piece of information that is known is the radius of gap junctions. Current literature assumes that the diameter of mammalian gap junctions is approximately 1.5 nm (15 Å; 19). Pore diameter is a function of the structure of the gap junction and is highly dependent on the connexin sub-components. As a result, the determination of a suitable pore diameter for gap junctions will be highly dependent on the cell type and species being investigated. From channel conductance experiments, gap junction pore diameters range from a minimum of 8 Å for connexin45 to 24 Å for the human connexin37 (31). For this study we will assume an average pore diameter of 15 Å with the understanding that this may be too small or too large for the MDCK cell line.

With this information, we are able to calculate the temperature range, and hence ice crystal size, whereby the propagation of intracellular ice will be possible. In Figure 5.4 we have plotted the capillary freezing point depression as a function of contact angle for 3 different pore radii. For a pore radius of 7.5 Å, and a contact angle of >80°, Equation 5-2 indicates that ice will be able to propagate between cells if there is approximately 10 degrees of supercooling. If the pore radius is increased to 12 Å or the contact angle is >85° then the required degree of supercooling will be approximately 5°C. These results are in strong agreement with those proposed by Mazur (21,22,23). Experimental verification of these results is required.

It must be emphasized that the Kelvin equation has only been validated down to pores of 4 nm (40Å; 14). With decreasing pore diameter, there may be significant deviations in the microscopic interactions of water and the pore as one approaches molecular dimensions. For gap junctions with a radius of 7.5 Å, less than 5 water molecules will fit across the entire pore. Clearly, the use of Equation 5-2 to model the effect of temperature on ice crystal size for pores of <40 Å may not be accurate. Further studies are required to validate Elliott's equation down to 1 nm.

5.4 ICE GROWTH AND GAP JUNCTIONS – EXPERIMENTAL EVIDENCE

If intracellular ice propagates between adjacent cells via gap junctions, then according to Mazur and Elliott's equations, there must be a defined temperature where the equilibrium curvature of an ice crystal is too large to pass through the pores. Using directional solidification, Berger and Uhrik were able to demonstrate that such a temperature does exist, but did not provide sufficient information to calculate the temperature (7). To experimentally validate Mazur's pore theory we will look at the formation of intracellular ice in two different cell lines that do and do not form gap junctions.

V-79W and MDCK cells were cultured to confluency on glass cover slips over 3 d at 37°C in supplemented media. Under the culture conditions used in this study, gap junctions were not identified in the V-79W monolayers (3,5,34), but do form in MDCK monolayers (34). The confluent monolayers were stained with 12.5 μM SYTO 13 to assist in the detection of intracellular ice (1,4). The glass cover slips were transferred to a convection cryomicroscope and cooled at 25°C/min to a predetermined experimental temperature were ice was initiated with a cold copper probe. The cooling power of the cryomicroscope was sufficient to remove the latent heat of fusion within 0.5 s after ice initiation. The occurrence of intracellular ice formation was indicated by a sudden darkening of the cytoplasm (11,20) or the disruption of the fluorescent staining (1,4). The entire freezing procedure was videotaped for later analysis.

The incidence of intracellular ice formation in V-79W and MDCK confluent monolayers is shown in Figure 5.5. For V-79W cells, there is a defined increase in the incidence of IIF between –5 and –11°C. The temperature where 50% of the cells in the monolayer have formed intracellular ice was –7.3°C. This data is consistent with results already published for V-79W monolayers (1,5,17). The incidence of intracellular ice formation was significantly different for the MDCK monolayers, with a 50% temperature for MDCK monolayers of –1.9°C. At temperatures less than –3°C there was complete formation of intracellular ice in all of the constituent cells of the confluent monolayer. At temperatures greater than –3°C the incidence of intracellular ice decreased dramatically. This point of discontinuity has not been previously documented for MDCK confluent monolayers.

It is of interest that the MDCK confluent monolayers can form intracellular ice at relatively high subzero temperatures. With only 1.5° of supercooling, more than 20% of the constituent cells formed intracellular ice. The cause of this increased incidence of intracellular ice formation is unknown. However, it is unlikely that this is a result of efficient nucleating agents within the MDCK cell cytoplasm, as MDCK cells in suspension respond the same as V-79W (1) and other cultured cells. It would seem then that the morphology of these cells might contribute to the increased susceptibility to intracellular freezing. Both confluent monolayers and single attached cells display an increased incidence of intracellular ice at high subzero temperatures compared to hamster fibroblasts (1,2).

While Figure 5.5 provides a summary of the number of cells that form intracellular ice, it does not give any indication of the mechanism by which the ice forms. From the videotapes it was possible to conduct a frame-by-frame analysis of the freezing process to examine the pattern of ice formation and intercellular propagation. In Figures 5.6 to 5.8 we provide a schematic view of the pattern of IIF of the V-79W and MDCK monolayers. In the V-79W monolayers we observed the non-random propagation of ice between adjacent cells (Figure 5.6) that was first documented by Acker and McGann (1,3,5). Initially there was a random dispersion of cells that form intracellular ice. This was followed by the induction of ice between neighbouring cells.

The freezing pattern of MDCK confluent monolayers depended on the temperature of nucleation. Below –3°C, intracellular ice formation occurred in a very well defined, wave-like manner (Figure 5.7). Shortly after the extracellular ice front passed through the field of view, intracellular ice formed, and nucleation of adjacent cells followed in the same direction. This pattern of intracellular ice formation has been observed in a number of tissues (7,27,29,30,33), including MDCK monolayers (1,2). The pattern of intracellular ice formation was significantly different when the nucleation temperature was above –3°C (Figure 5.8). In MDCK monolayers that are nucleated above –3°C, the pattern of ice formation strongly resembles the pattern observed in V-79W monolayers. Ice formed initially in a number of single cells in the sample and then propagated to adjacent cells.

To further examine the phenomenon of intracellular ice formation in V-79W and MDCK cells we extracted the number of independent nucleation sites from the microscopy data set. An independent nucleation site was defined as a cell that froze intracellularly without any interaction with an already frozen adjacent cell. We calculated the number of independent nucleation sites and compared it to the total number of cells in the sample that form intracellular ice to get a percentage. A high value indicates that there were a large number of cells that formed intracellular ice independent of a frozen neighbouring cell. A low value indicates that there was a high degree of interaction between adjacent cells.

There was a significant difference in the percentage of independent nucleation sites for V-79W and MDCK confluent monolayers (Figure 5.9). For V-79W monolayers the percentage of independent sites starts high and decreases non-linearly with decreasing temperatures. As the temperature decreases there are fewer independently frozen cells compared to higher subzero temperatures. The percentage of independent nucleation sites asymptotically approaches some non-zero value at high subzero temperatures. This suggests that even at temperatures where all of the cells will form intracellular ice, that nucleation will originate from a small number of independent sites. In the MDCK monolayer, we saw that there was a defined discontinuity in the percentage of independent nucleation sites as a function of nucleation temperature. At temperatures above -3°C, there was a linear decrease in the number of independent nucleation sites with decreasing temperature. At –3°C or below, there were no independent nucleation sites. This is a result of the definition we used for independent nucleation sites. In MDCK monolayers that are nucleated below –3°C, nucleation occurs outside the field of view, and all that is observed is the wave-like propagation of intracellular ice (Figure 5.7). In this case there are no observable independent nucleation sites and hence the value reported is zero.

Taken together, this data demonstrates the discrete differences that exist in the formation of intracellular ice in V-79W and MDCK confluent monolayers. The incidence and pattern of intracellular ice formation in these two experimental systems are distinct. By carefully comparing these two experimental model systems we can extract critical information on the mechanism of intracellular ice formation in tissues and tissue model systems.

While there is a wealth of information that can be gained from this data, we will focus solely on the discontinuity that occurs at –3°C in MDCK monolayer as it relates directly to our study on cell-cell propagation. In MDCK monolayers, a cell line that does form gap junctions, there is a defined transition in the incidence and pattern of intracellular ice formation at –3°C. At temperatures below –3°C, intracellular ice is observed in all cells, and the wave-like propagation suggests that induction occurs with relative ease. However, above –3°C, IIF does not occur as readily nor does it occur in the same manner. Above –3°C, the MDCK monolayer responds in much the same way as the V-79W monolayer; a cell line that does not form gap junctions. Clearly, there are a number of differences in the morphological and physiological characteristics between these two cell types that could affect the low temperature response of these confluent monolayers. However, it is the author's belief that the differences observed between the incidence of IIF in V-79W and MDCK are a direct result of the presence or absence of gap junctions.

If intracellular ice propagates between adjacent cells via gap junctions, then according to the Elliott equation, there must be a defined temperature where the ice crystal of equilibrium curvature is too large to pass through the pores. From studies with MDCK and V-79W monolayer we conclude that this temperature is approximately –3°C. This temperature is significantly higher than the –10°C that has been suggested to be the minimum temperature that would allow ice to propagate through membrane pores (21,22,23,26).

5.5 CONCLUSIONS

As has been stated, for intracellular ice to propagate between adjacent cells in tissues or tissue models, there must be a mechanism for this to occur. It has been proposed that pores in the membrane, or gap junctions, can facilitate the passage of ice between neighbouring cells. For this to occur, the radius of the ice crystal must be of a sufficiently small size. According to the Elliott equation, as temperature increases, the equilibrium ice crystal radius increases, and hence the propagation of ice between cells must be temperature dependent. In this study we have coupled the predictive value of an equation that is analogous to the Kelvin equation with experimental evidence to support the proposed theory (1,7) that gap junctions can facilitate the intercellular propagation of intracellular ice.

The observations and data presented herein provide experimental support for the proposed pore theory of intracellular ice formation. Using a more biologically relevant and thermodynamically correct form of Mazur's equation, we determined that the temperature range for intracellular ice propagation via gap junctions is highly dependent on the pore size and the contact angle. As the contact angle approaches 90° for a pore with a radius of 7.5 Å, the temperature required for ice propagation approaches the freezing point of the solution. In the range of 75-85°, and a pore radius between 5 and 12 Å, Elliott's equation predicts that the temperature required for ice propagation will be between -2 and -22°C.

Experimentally we observed a significant change in the behavior at –3°C that we believe is a result of the inhibition of gap junction-facilitated ice propagation. As this temperature is in the range predicted by Elliott's equation, it provides strong, indirect evidence to support the pore theory of ice propagation.

A further advantage of coupling experimental data with the Elliott equation is the ability to verify the assumptions made in the calculations. The relatively high subzero temperature at which ice propagation via gap junctions was inhibited provides supportive evidence that the contact angle is in fact closer to 90° than 0°. For the radius of pores that are biologically relevant (4 – 24 Å), the contact angle that would result in a freezing point depression of 3°C must be greater than 85° (Figure 5.3). This calculated contact angle is in agreement with Mazur's assumption on the contact angle required for ice propagation (21,22,23).

By combining theoretical predications and experimental data, this study provides strong evidence to support the concept that intracellular ice formation can propagate between cells via gap junctions. Understanding the mechanism by which intracellular ice can pass from one cell to another is critical to developing mechanistic and mathematical models to predict the occurrence of IIF. While this study provides an explanation for the ice propagation seen in tissues and tissue systems that have gap junctions, it does not advance our understanding of ice propagation in systems that do not have gap junctions. Clearly, there is further work required to understand the complex low temperature response of tissue systems.

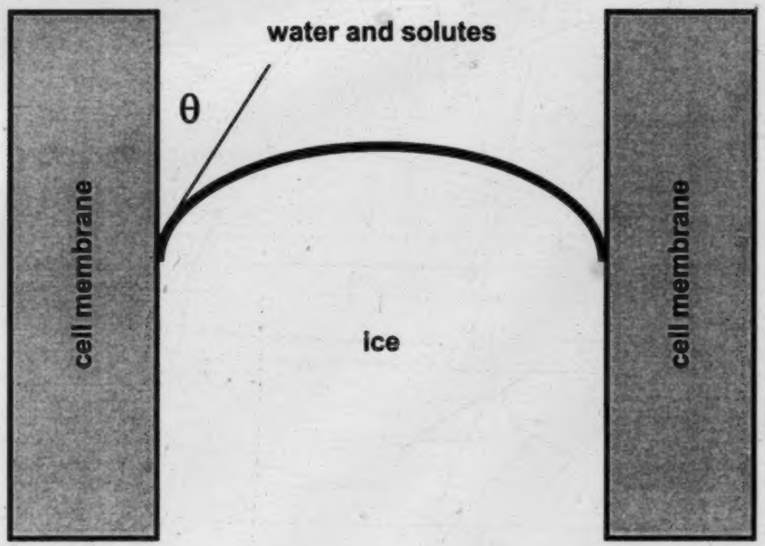
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Figure 5.1 – Schematic representation of ice propagation through a transmembrane pore.

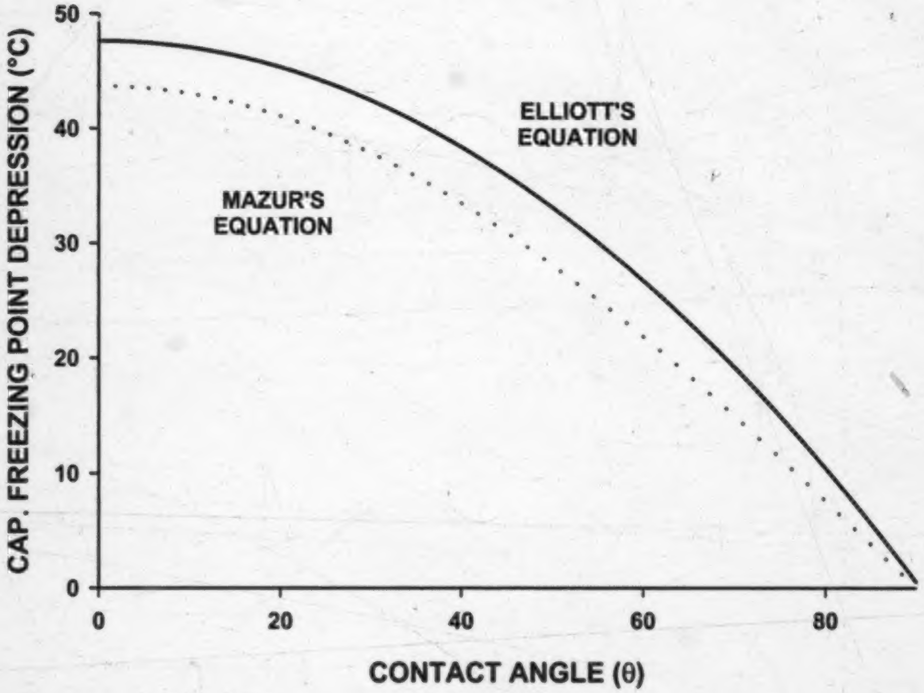

Figure 5.2 – Error associated with the use of Mazur's approximate equation. Mazur's equation is compared to the equation derived by Elliott (Equation 5-2). The capilliary freezing point depression is calculated as a function of contact angle for a pore radius of 7.5 Å.

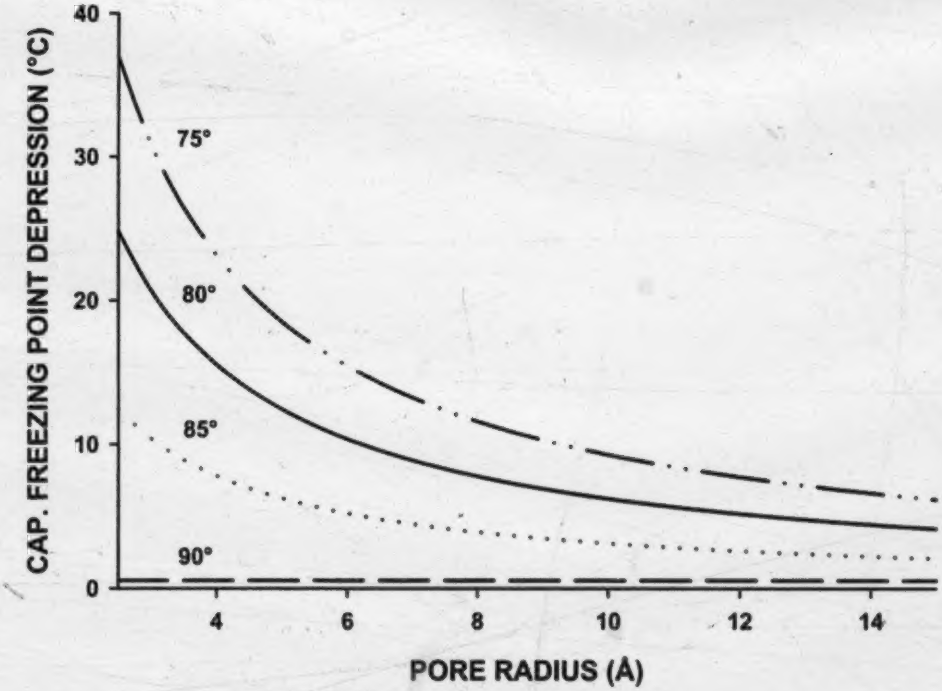

Figure 5.3 – Capilliary freezing point depression as a function of pore radius for various contact angles.

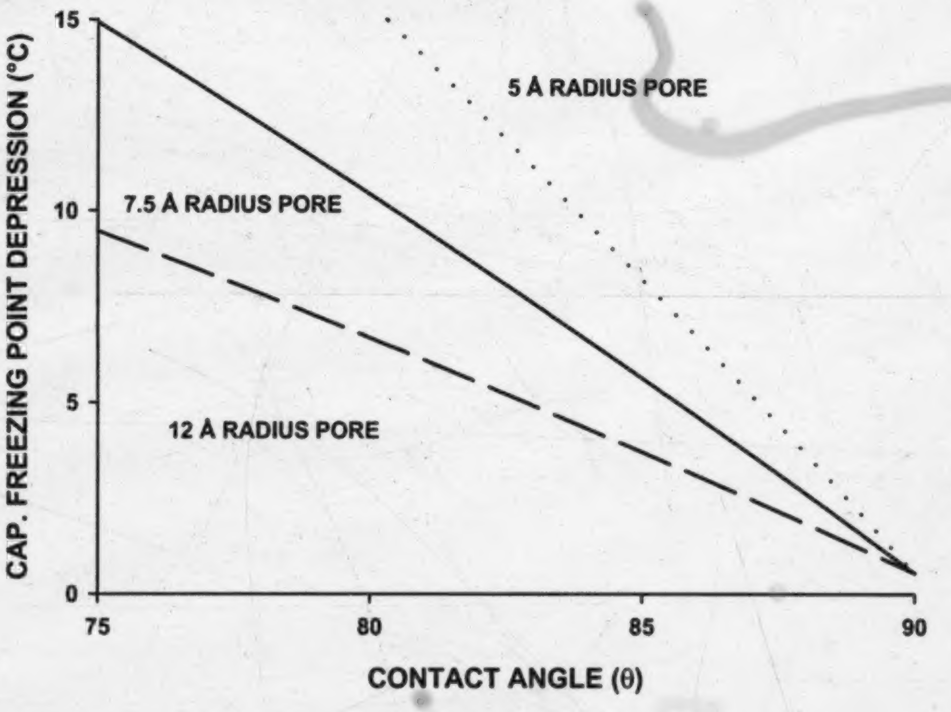

Figure 5.4 – Capilliary freezing point depression as a function of contact angle for three pores with defined radii.

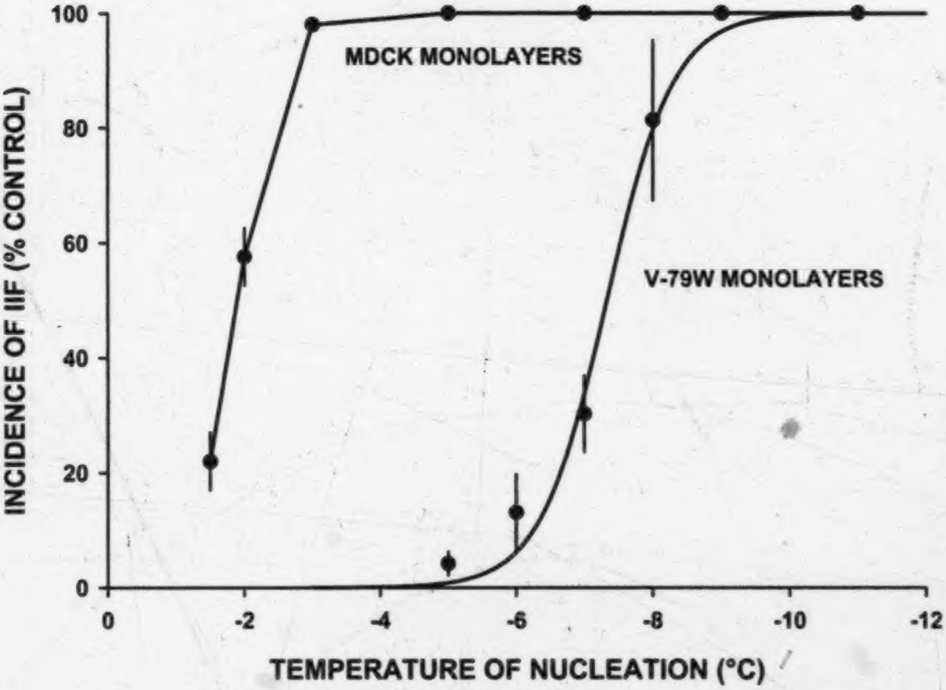

Figure 5.5 – Incidence of intracellular ice formation in V-79W and MDCK confluent cell monolayers. Mean ± SEM (n=3).

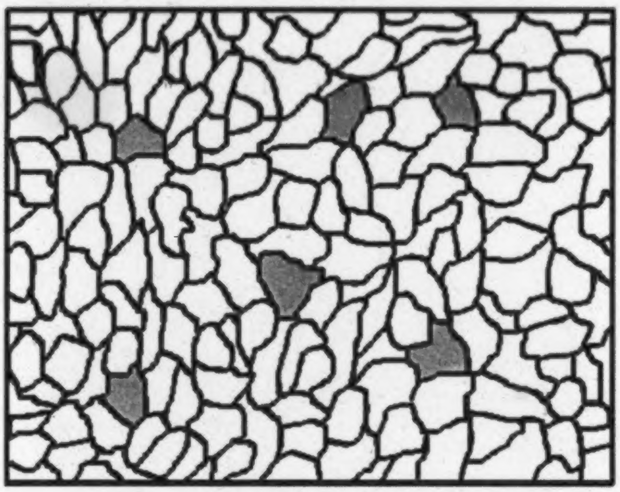

Figure A

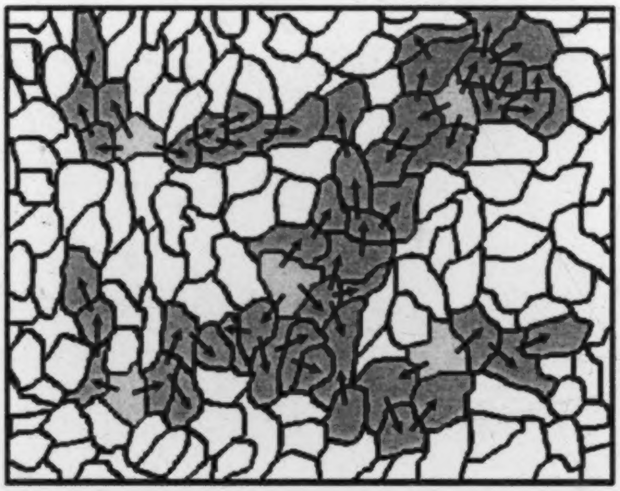

Figure B

Figure 5.6 – A schematic representation of the pattern of intracellular ice formation in V-79W confluent cell monolayers. Ice forms initially in a random number of cells that are dispersed throughout the monolayer (A). This is followed by the non-random propagation of intracellular ice between adjacent cells (B).

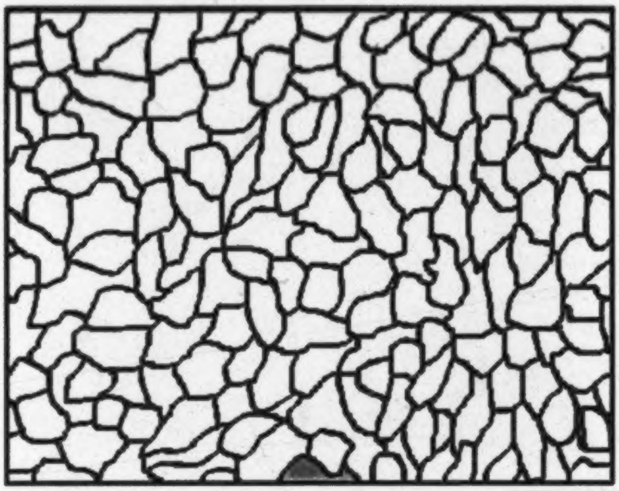

Figure A

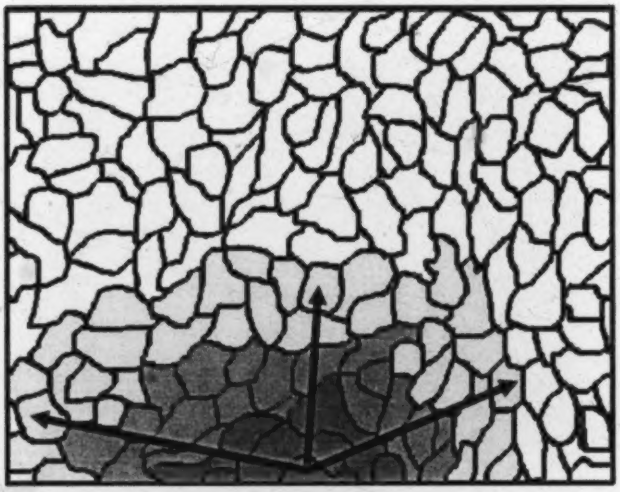

Figure B

Figure 5.7 – A schematic representation of the pattern of intracellular ice formation in MDCK confluent cell monolayers frozen below -3°C. As extracellular ice front passess through the field of view, intracellular ice will form in a cell (A). This is followed by the propagation of intracellular ice between adjacent cells in a wave-like pattern (B).

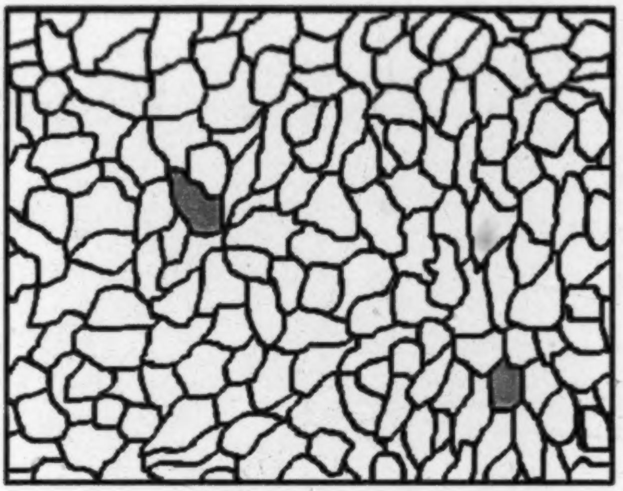

Figure A

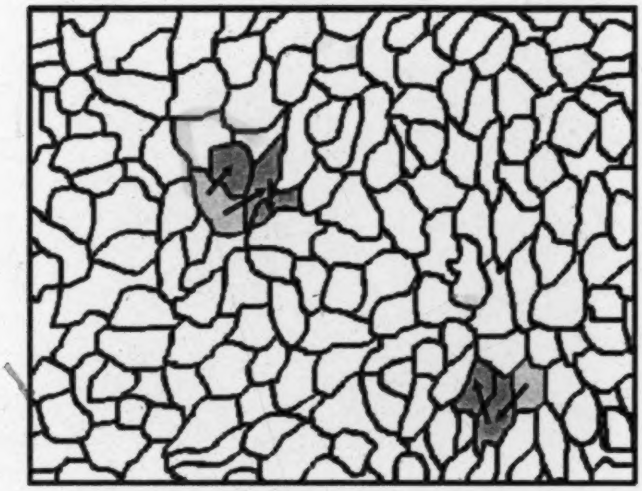

Figure B

Figure 5.8 – A schematic representation of the pattern of intracellular ice formation in MDCK confluent cell monolayers frozen above -3°C. Ice forms initially in a random number of cells that are dispersed throughout the monolayer (A). This is followed by the propagation of intracellular ice between adjacent cells (B). This pattern is identical to that observed in V-79W confluent monolayers.

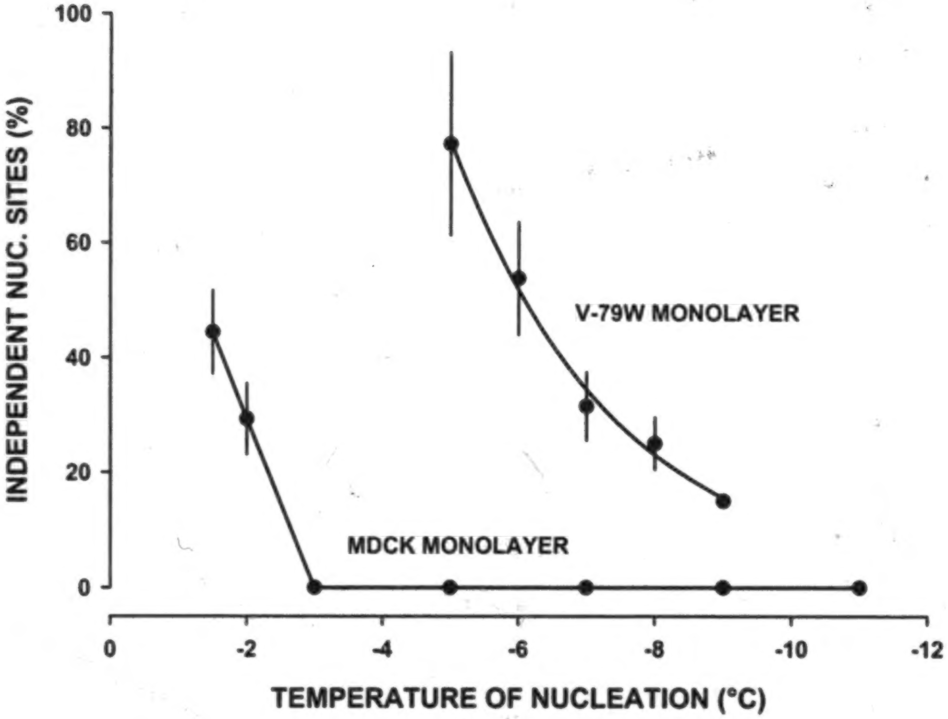

Figure 5.9 – Percent independent nucleation sites as a function of the temperature of nucleation for V-79W and MDCK confluent monolayers. An independent nucleation site is defined as a cell that forms intracellular ice without any contact with an already frozen cell. The percentage of independent nucleation sites is calculated as the total number of independent sites divided by the total number of cells that form intracellular ice. Mean ± SEM (n=6).

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CHAPTER SIX

CONCEPTUAL MODEL FOR INTRACELLULAR ICE FORMATION IN CONFLUENT MONOLAYERS

6.1 INTRODUCTION

Understanding the freezing response of tissues is an essential step in the development of successful cryopreservation protocols. Current efforts to cryopreserve tissues and organs rely on ideas and models developed from the biophysical and physiological data collected from cellular systems. The failure of this approach is likely a result of the complexity of organized tissues and the lack of information available on the tissue-specific elements involved in freezing. The diversity of cell types and cell densities as well as the morphological differences complicates the determination of the osmotic and thermal state of the system (18,25,29). For the successful development of methods for the cryopreservation of tissues and organs, current ideas and theories regarding the freezing response of tissues must be challenged. Only after gaining a more complete understanding of the intricate factors involved in the low temperature response of tissues will we be able to move forward.

A poorly understood physical phenomenon that readily occurs during the freezing of living systems is intracellular ice formation (IIF). The ability to predict the probability of IIF for a given freezing protocol has met with limited success because of an inadequate understanding of the mechanism(s) by which intracellular ice damages cells. The search for a model that can accurately predict the occurrence of IIF has been motivated by the potential use of this model in the design of cryopreservation protocols. The basis for all of the current models for the prediction of IIF has been Mazur's physiochemical model for water transport (21). Numerous phenomenological (23,30,31,32) models have been developed which use statistical approaches and an understanding of the conditions surrounding the cell just prior to freezing to predict the likelihood of intracellular freezing. Mechanistic models have also been developed, which incorporate the mechanisms of membrane failure (28) and surface-catalyzed nucleation (37) to predict the occurrence of IIF. These two approaches, phenomenological and mechanistic, while fundamentally different in design, have been shown to give relatively similar end results (17). Each model is able to predict with some degree of certainty, the degree of intracellular ice formation in a cellular system under very controlled conditions. However, the application of these methods to modeling the formation of intracellular ice formation in tissue model systems has been unsuccessful. Current models do not give consideration to the unique conditions for intracellular ice formation in tissues (18,39).

Understanding the mechanism of intracellular ice formation has important implications in the development of protocols for the cryopreservation of tissues and tissue model systems. Detailed mathematical models have been developed which attempt to address the response of tissues to freezing. These have included: the Krough cylinder model (33); the network thermodynamic model (11,13); the modified Krough cylinder model (9); and most recently the compartmental model (36). The weakness in using these techniques to predict the low temperature response of a tissue is that they are all based on the biophysical data collected from cell suspensions. While these models address the physical changes involved in scaling from a cell to a tissue, none of them consider the unique properties of tissues and the effect that these have during freezing and thawing. Therefore, a more detailed understanding of the mechanism by which tissues are damaged would greatly enhance the efficacy of using mathematical modeling.

Avoiding intracellular ice formation has been a common strategy in the cryopreservation of cell suspensions, but recent studies with tissue model systems suggest that this may be much more difficult during tissue cryopreservation. It has been demonstrated in cultured hepatocytes (10,16), fibroblasts (1,4) and keratinocytes (5) that there is a significant increase in IIF at lower cooling rates and at higher subzero temperatures than occurs in the same cells in suspensions. It has further been reported that cell-cell contact can facilitate the nucleation of ice between adjacent cells (1,4,8). These observations have led to the conclusion that intracellular ice formation occurs much more readily in tissue systems as a result of the unique morphology and physiology (1). Avoiding intracellular ice therefore becomes much more difficult and in some cases, may not be possible. If intracellular ice is inevitably lethal, then these results are not very encouraging. We have previously demonstrated that intracellular ice is not necessarily lethal (chapter 2), and, in some cases, may be protective (chapter 3).

The purpose of this study is to expand our current understanding of intracellular ice formation in confluent monolayers into a conceptual, phenomonological model. Through the process of developing this model, we will bring together data that has been previously described. This will include information on the role of the cell plasma membrane, the effect of cell-cell propagation, and the freezing response of confluent monolayers. Experimental evidence will be used to validate the assumptions and conclusions. This work will represent the first concerted effort to develop a descriptive model for the formation of intracellular ice in two-dimensional tissue systems.

6.2 THE MODEL

From the data that has been presented in this thesis, it is clear that intracellular ice formation in cultured cells is a complex process. Before we address the formation of intracellular ice in confluent monolayers, we will summarize our understanding of IIF in single attached cells. In Figure 6.1 we see a schematic representation of intracellular ice formation in single attached cells. During freezing ice will initially form extracellularly and the cell cytoplasm will remain supercooled. The probability that a cell will form intracellular ice is dependent on the temperature (Table 4.1 and 4.2), with a larger incidence of intracellular ice occurring at higher degrees of supercooling. Commensurate with the formation of intracellular ice is a disruption of the cell plasma membrane (Figure 4.1 and 4.2). While membrane damage may be the cause of (12,23) or results from (6,28,35) the formation of intracellular ice, this damage occurs during freezing (Figure 4.8 and 4.9). Post-thaw, the formation of intracellular ice in single attached cells and the corresponding disruption in the plasma membrane result in cell death (Figure 3.1 and 3.2). As the presence of intracellular ice is closely linked to membrane damage and cell death, avoiding the conditions whereby intracellular ice formation will occur, can enhance cell recovery in single attached cells. The mechanism of IIF in single attached cells is very similar to what has been concluded from work with single cells in suspension (12,17,27).

The formation of intracellular ice in confluent monolayers is much more complex than what we have proposed for single attached cells. There are two defined stages to intracellular ice formation in confluent monolayers: an initial formation and the subsequent step-wise propagation of intracellular ice that occurs between adjacent cells (Figure 5.6 to 5.8). In confluent monolayers, IIF is highly dependent on the presence and type of cell-cell junctions. We will consider the formation of intracellular ice in confluent monolayers with and without gap junctions. A schematic representation of the descriptive model for intracellular ice formation in confluent monolayers is shown in Figure 6.2 and 6.3.

Let us consider the formation of intracellular ice in confluent monolayers that do not have gap junctions (Figure 6.2). As the confluent monolayers are cooled below the freezing point of the solution, ice will form extracellularly. In the absence of gap junctions, there is an initial formation of intracellular ice in cells that are randomly dispersed throughout the monolayer (1,2,3). These initial cells that form intracellular ice will not have any adjacent cells with intracellular ice present. Membrane damage will occur during the freezing of these independent cells (Figure 4.12 and 4.14). The mechanism by which the membrane is damaged is unknown, as it is unclear whether intracellular ice is the cause of (12,23) or results from (6,28,35) damage to the plasma membrane. Further experimentation is required to determine the sequence of events before a more definitive statement can be made on the mechanism of IIF in the initial cells of a confluent monolayer.

Following the initial formation of ice in a few cells randomly scattered throughout the monolayer, propagation of intracellular ice between adjacent cells occurs (Figure 5.6). As there are no pores through which ice can propagate, intercellular ice growth may occur as a result of surface-catalyzed nucleation (1,37,38,39) or some other unknown process. The formation of intracellular ice in these adjacent cells does not result in damage to the cell membrane of most cells (Table 4.3). However, some do display a damaged plasma membrane following intracellular ice propagation (Figure 4.13). The probability of a constituent cell in a confluent monolayer surviving intracellular ice formation is dependent on the presence of an intact plasma membrane (Figure 2.3). The cells that initially form intracellular ice are killed as a result of the damage done to the plasma membrane. However, those cells that form intracellular ice via cell-cell propagation without damage to the membrane will survive.

With decreasing nucleation temperature there is a decrease in the number of independent IIF sites (Figure 4.14). At lower temperatures, the efficiency of cell-cell propagation must be greater than that at higher subzero temperatures. As the initial formation of intracellular ice is a stochastic process (1,3) and is strongly time dependent, at low temperatures cell-cell propagation may occur before the cell would freeze independent of a frozen neighbour. This results in fewer independent IIF sites (Figure 4.14), but an increase in the total incidence of intracellular ice formation in the monolayer (Figure 4.3). As membrane damage is linked to the initial formation of intracellular ice, as more cells form ice via cell propagation at lower temperatures, the total incidence of membrane damage will remain constant, or decrease (Figure 4.3).

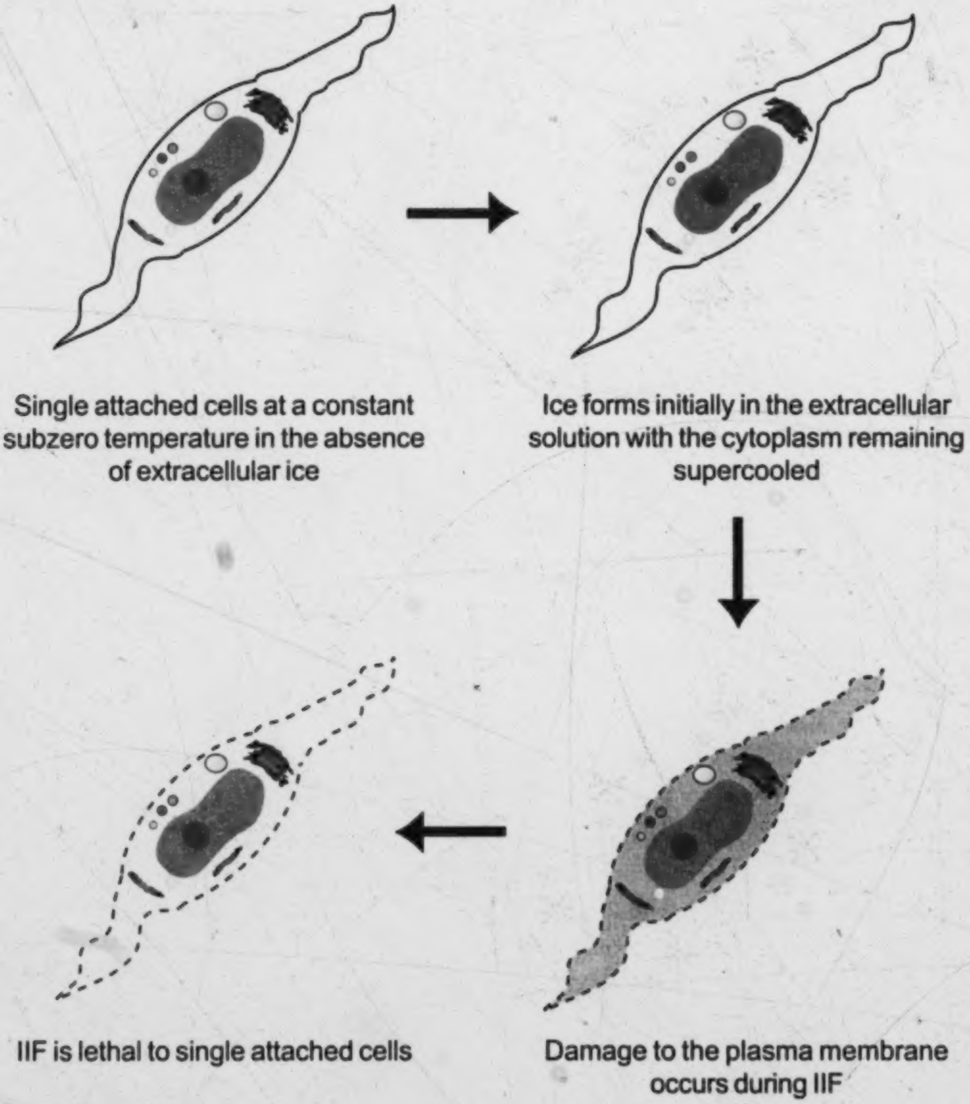
The mechanism of intracellular ice formation in confluent monolayers that do form gap junctions is slightly different than in the absence of gap junctions (Figure 6.3). As the confluent monolayer is cooled ice will form extracellularly at the freezing point of the solution while the cytoplasm will remain supercooled (12,22). In these monolayers there is an initial formation of intracellular ice that occurs. However, unlike monolayers that do not express gap junctions, this initial formation of ice in a single cell is sufficient to nucleate the entire monolayer. As gap junctions connect the cytoplasm of all of the constituent cells of the monolayer, intercellular ice propagation is easily facilitated (Figure 5.7). Complete formation of intracellular ice will therefore occur at much high subzero temperatures and low degree of supercooling where only a single cell is required to freeze intracellularly. As the mechanism of formation of intracellular ice is likely the damaging event (chapter 2), monolayers that express gap junctions are less likely to be damaged (chapter 3).

The propagation of intracellular ice via gap junctions provides a mechanism by which intracellular ice can form without the disruption in the plasma membrane. Maintaining plasma membrane integrity has been shown to be critical to the survival of cells in confluent monolayers (chapter 2). Unfortunately, some cells in the confluent monolayer display a damaged plasma membrane even if the formation of intracellular ice occurred via gap junction-facilitated cell-cell propagation. The number of cells with membrane damage is independent of temperature (Figure 4.4) and the cells are randomly dispersed throughout the monolayer (1,2). The mechanism whereby the presence of intracellular ice can result in damage to the plasma membrane is unknown. There are a number of proposed ideas as to the mechanism by which the presence of intracellular ice damages can damage a cell including: recrystallization (23,24), solution effects and thermal shock (15), osmotic injury (14,28), protein denaturation (19), and/or gas bubble formation (7,26,34). Further work is required to investigate this mechanism of cryoinjury.

At high subzero temperatures, where the radius of the ice crystal is too large to propagate through gap junctions, the mechanism of IIF is identical to that which occurs in confluent monolayers without gap junctions (Figure 5.8).

6.3 CONCLUSION

The purpose of this chapter has been to expand our current understanding of intracellular ice formation in confluent monolayers into a conceptual, phenomonological model. Using novel techniques to examine the role of the cell plasma membrane and the effect of cell-cell junctions on the formation of intracellular ice, we have demonstrated the ability to study intracellular freezing at a level not previously possible. This work clearly demonstrates that the freezing response of a cell in a confluent monolayer is much more complex than the response of a cell or single attached cell. By establishing a mechanism for the formation intracellular ice in two-dimensional tissue models, we have provided the foundation for the development of more effective mathematical models of tissue freezing. In the process we have presented experimental evidence which has challenged some of the long-standing ideas in cryobiology on the formation of intracellular ice. The results of this work are of theoretical interest to the mechanisms of IIF and of practical interest in the cryopreservation of tissues.



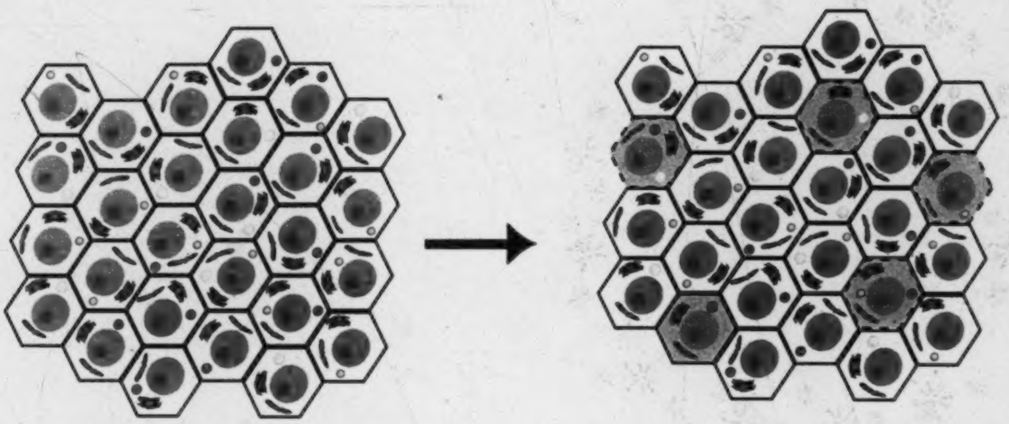
Single attached cells at a constant subzero temperature in the absence of extracellular ice

Ice forms initially in the extracellular solution with the cytoplasm remaining supercooled

Damage to the plasma membrane occurs during IIF

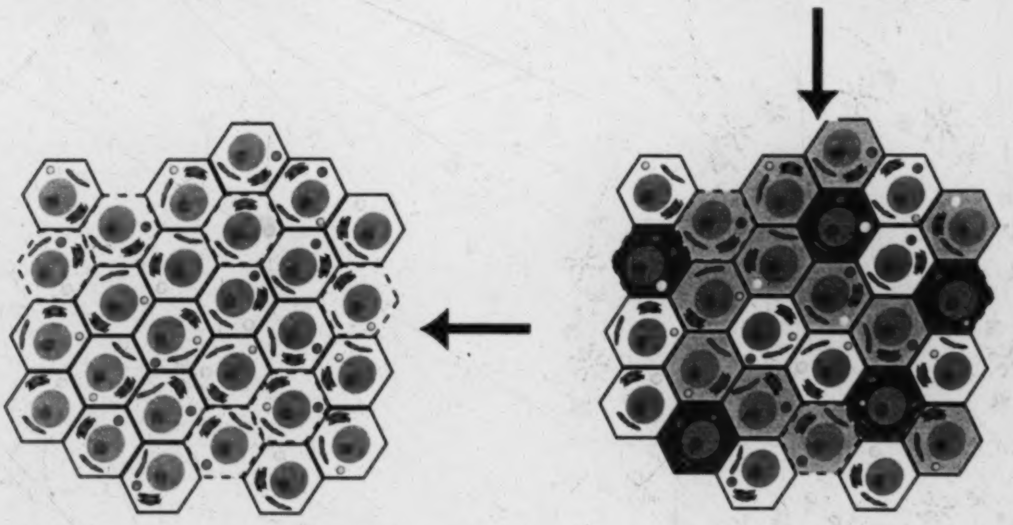
IIF is lethal to single attached cells

Figure 6.1 – Schematic representation of the model for intracellular ice formation in single attached cells.



Confluent monolayer supercooled at a constant subzero temperature in the absence of extracellular ice

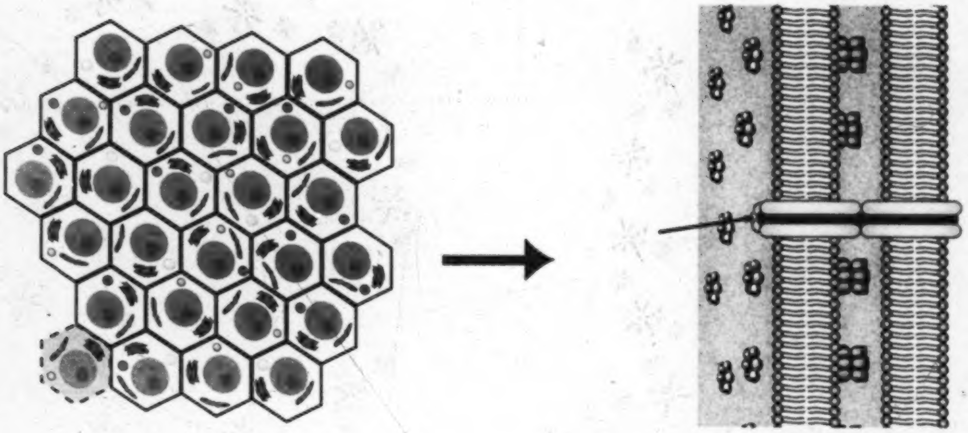
Initial formation of intracellular ice in randomly dispersed cells results in membrane damage



Lethal injury to confluent monolayers is correlated with membrane damage

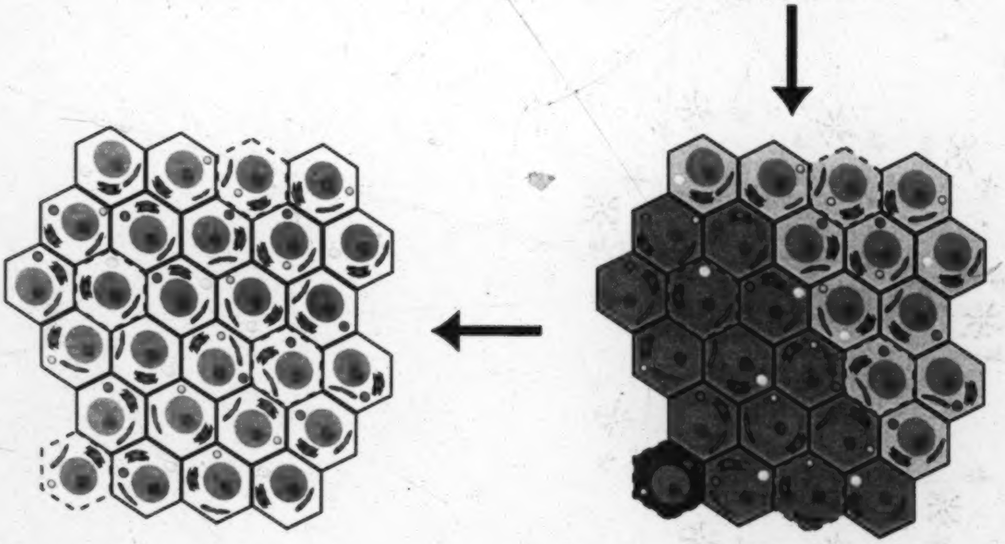
Non-random propagation of intracellular ice to adjacent cells; additional cell membrane damage

Figure 6.2 – Schematic representation of the model for the formation of intracellular ice in confluent monolayers without gap junctions.



Initial cell that forms intracellular ice will have a damaged plasma membrane

At low temperatures, intracellular ice can propagate between adjacent cells via gap junctions



Lethal injury to confluent monolayers is correlated with membrane damage

Intercellular ice propagation between all cells with a few additional cells suffering membrane damage

Figure 6.3 – Schematic representation of the model for intracellular ice formation in confluent monolayers with gap junctions.

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CHAPTER SEVEN
CRYOPRESERVATION AND INTRACELLULAR ICE FORMATION

7.1 INTRODUCTION

The successful cryopreservation of a wide variety of cell types has been a result of the development of novel techniques to minimize cell damage. In cell suspensions, damage to cells can be caused by both intracellular freezing and exposure to high concentrations of solutes (25). Current cryopreservation protocols emphasize a reduction in the amount of ice formed (28). During slow cooling, a reduction in extracellular ice can limit the concentration of extracellular solutes (24,25) and hence the degree of damage. Similarly, during rapid cooling, if intracellular ice formation can be inhibited or limited, then the scale of damage done to the cells can be significantly reduced (24,25). Avoidance of ice formation has therefore been the main technique used in the cryopreservation of cellular systems.

In 1949, Polge, Smith and Parkes introduced the idea of using chemical compounds to enhance the survival of frozen biological material (31). With the addition of glycerol to their samples, they demonstrated a significantly greater proportion of viable bovine spermatozoa after thawing from -79°C (31). A variety of chemical compounds, or cryoprotectants, have since been shown to colligatively effect the physical properties of solutions (26,27). Cryoprotectants are capable of reducing the amount of ice formed in a concentration-dependent manner (28). However, it quickly became known that high concentrations of cryoprotectants had detrimental toxic (13,24,25) and osmotic effects (24,29,40) on cells. Innovative protocols for the addition and removal of these cryoprotectants were therefore developed in efforts to avoid these damaging effects.

The traditional approach to the cryopreservation of cells has therefore been to balance the toxic effects of high concentrations of solutes with cooling rates slow enough to avoid IIF, but rapid enough to minimize exposure to the cryoprotectants. This method is based on the principle of preventing the formation of intracellular ice, and assumes that any intracellular ice is lethal to the cells. Through the extensive study of the intricate elements of a cells response to cooling and warming at different rates and under various conditions, a comprehensive understanding of the effects of low temperatures on cellular systems has been achieved. This information has facilitated numerous productive approaches for the cryopreservation of a wide variety of cell types.

Although successful cryopreservation can be achieved for a variety of cell types isolated in suspension, when *in situ*, these cells are severely damaged. For example, cells from the heart (5), the liver (17) and the kidney (11) can recover partial viability upon freezing while in suspensions. However, attempts to freeze these whole organs have proved unsuccessful (20,30). Additional factors must affect the *in situ* cells response to low temperatures resulting in the increased damage. The diversity of cell types and cell densities as well the morphological differences between cells and tissues complicates the determination of the osmotic and thermal state of the system (21,30). The presence of cell junctions in multicellular systems has also been proposed as a contributing factor to the enhanced sensitivity of tissues to freezing injury (1,2,3,6,7). Elucidating the role that these factors have in the low temperature response of tissues is an important step in the design and implementation of successful cryopreservation protocols.

Techniques for the cryopreservation of tissues have taken two distinct paths. The traditional approach has been to use high concentrations of cryoprotectant and rapid cooling rates to reduce or eliminate ice formation. By balancing the toxicity of the cryoprotectants and osmotic stresses imposed during the addition and removal, vitrification of tissues and organs has been actively pursued. Recent reports of the vitrification of rabbit kidneys (23) indicates that this technique may be feasible for the preservation of whole organs. However, there still exists numerous physical limitations to this technique which must be resolved (14). The second method for the cryopreservation of tissues couples slow cooling and controlled equilibration of cryoprotectants with rapid warming rates. This technique has been shown to be relatively successful in the preservation of arteries (36) split-thickness skin (42), pancreatic islets (32) and hepatocyte monolayers (9,38,41). Under these conditions, thermal and mechanical effects become important factors in the practical application of this technique (19). Clearly, the current trend for the cryopreservation of tissues is to minimize the effects of extracellular ice and avoid the formation of intracellular ice.

The application of classic freezing techniques to the cryopreservation of tissues and tissue models assumes that intracellular ice is lethal. This conclusion has been based largely on observations made in cellular systems, with very little corroborating evidence from tissue systems. In fact, there is strong evidence to suggest that rapid freezing and the formation of intracellular ice in confluent monolayers and tissues is innocuous. Early studies with rabbit corneal tissue (18), skin (10,22,35), and tumor (8,34,35) and plant (33) tissue have concluded that intracellular ice is innocuous. Recent work with split-thickness skin and kidney slices has demonstrated that rapid cooling can result in high degree of recovery (12,43). While the authors suggest that this is a result of vitrification, microscopic evidence (43) clearly demonstrates the presence of intracellular ice. Finally, one of the most dramatic examples of the innocuous nature of intracellular ice was demonstrated during the freezing of the antarctic nematode *Panagrolaimus davidi* (39). Following the complete formation of intracellular ice throughout the body of *P. davidi* and subsequent warming, these animals were able to grow and reproduce in culture. As all of these cases demonstrate that rapid cooling and IIF can result in significant post-thaw recovery, it must be concluded that intracellular ice is not always lethal.

The universality of the presumption that intracellular ice is lethal in cells and tissues has recently been challenged by the results from a number of studies (1,4,16). Using a novel method to prevent ice crystal growth and membrane damage, Fowler and Toner demonstrated that large quantities of intracellular ice can be tolerated by erythrocytes (16). In confluent cell monolayers, IIF has also been shown to occur without any detrimental effects on the overall cell viability (chapter 2). A proposed mechanism by which intracellular ice can form in confluent monolayers and remain innocuous suggests that the plasma membrane is the critical site of damage (chapter 2 and 4). As it is possible to form intracellular ice in confluent monolayers without disrupting the plasma membrane (4, chapter 4), the possibility of using rapid freezing and the formation of intracellular ice in cryopreservation exists.

The purpose of this study is to establish the feasibility of using intracellular ice as a cryoprotectant in the cryopreservation of tissue systems.

7.2 MATERIALS AND METHODS

Cell Culture

Two cell lines were used to investigate the use of intracellular ice formation in cryopreservation. The first was the Madin Darby Canine Kidney (MDCK; CCL34 ATCC) epithelial cell line. These cells were grown at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential media (MEM) supplemented with 10% v/v fetal bovine serum (all components from GIBCO Laboratories, Grand Island, NY). Cells were kept in tissue culture flasks (25 cm^2; Corning Glass Works, Corning, NY) and harvested by exposure to a 0.25% trypsin-EDTA solution (GIBCO) for 10 min at 37°C. The MDCK cells were re-suspended in supplemented MEM to obtain cell suspensions and then plated on sterilized cover slips (12 mm circle, Fisher Brand) at a concentration of $2x10^4$ cells/mL. The cover slips were grown in petri dishes in an incubator for 12 h to allow the cells to attach (single attached cells) and 3 d to allow the growth of a confluent monolayer.

The second cell line was the V-79W line of Chinese hamster fibroblasts. Chinese hamster fibroblasts (V-79W) were incubated at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential medium (MEM) with Hanks' salts, 16 mmol/L sodium bicarbonate, 2 mmol/L L-glutamine and 10% fetal bovine serum supplemented with antibiotics (penicillin G (50 units/mL), streptomycin (50 µg/mL)) (all components from GIBCO Laboratories, Grand Island, NY). Cells were grown in tissue culture flasks (25 cm^2; Corning Glass Works) and harvested by exposure to a 0.25% trypsin solution (GIBCO) for 10 min at 37°C. The fibroblasts were resuspended in supplemented MEM. Sterilized cover slips (12 mm circle, FISHER Brand) were placed in a petri dish (FISHER Brand, 100 x 15 mm) and covered with 15 mL of supplemented MEM containing $3x10^5$ cells. The petri dishes were incubated for 12 h to allow the cells to attach (single attached cells) and 3 days to allow the growth of a confluent monolayer.

Viability Assessment

AlamarBlue™ (Biosource International, CA) was used to assess the overall metabolic activity of the confluent monolayers post-thaw (Appendix A). AlamarBlue was added to tissue culture media (5% v/v) and the single attached cells (MDCK and V-79W) were incubated in this solution for 24 hours at 37°C. Confluent monolayers (MDCK and V-79W) were incubated for 12 hours at 37°C in 5% alamarBlue. An aliquot of the media was removed and measured on a spectrophotometer (570-600 nm; UVmax, Molecular Dynamics, CA). Incidence of metabolic activity was calculated as the mean percent difference in reduction between the experimental samples and the controls using equation A-2 and A-3. Assessment of metabolic activity has been shown to correlate well with other viability assessments including membrane integrity and clonogenic function (Appendix A).

Standard Freezing Protocol

Madin-Darby canine kidney (MDCK) epithelial cells and V-79W hamster fibroblasts were either used as single attached cells or grown to confluency on glass coverslips. The cover slips containing a confluent monolayer were placed in 15 x 45 mm glass tubes (Kimble Glass Inc.) and placed on ice for 5 min. Following this incubation at 0°C, the samples were immersed in an alcohol bath at –5°C and allowed to cool for 5 min. Extracellular ice formation was induced in the samples using cold forceps. The bath was then cooled at 1°C/min to –40°C where the samples were held for 5 min. All samples were then rapidly warmed in a 37°C water bath and incubated in a 5% alamarBlue solution at 37°C. Metabolic activity was then assessed. Unfrozen samples served as positive growth controls.

Standard Freezing Protocol with Cryoprotectant

MDCK and V-79W single attached cells and confluent monolayers were subjected to the standard freezing protocol with addition of 10% v/v DMSO (Cryoserv, Tera Pharmaceuticals Inc., Buena Park, CA). The cover slips containing the cells were placed in 15 x 45 mm glass tubes and placed on ice for 5 min. Prior to freezing, 500 µL of a 10% DMSO solution was added to the glass tubes. The single attached cells and confluent monolayers were allowed to incubate with the DMSO for 20 min and then 450 µL of the solution was removed prior to freezing. Following this incubation at 0°C, the samples were immersed in an alcohol bath at –5°C and allowed to equilibrate for 5 min. Extracellular ice formation was induced in the samples using cold forceps. The bath was then cooled at 1°C/min to –40°C where the samples were held for 5 min. All samples were then rapidly warmed in a 37°C water bath, washed twice with tissue culture media to remove the cryoprotectant and incubated in a 5% alamarBlue solution at 37°C. Metabolic activity was then assessed. Unfrozen samples without DMSO served as positive growth controls. This freezing protocol is the standard technique used to cryopreserve V-79W and MDCK cell suspensions for routine laboratory work.

Modified Freezing Protocol with Intracellular Ice

MDCK and V-79W cells were either used as single attached cells or grown to confluency on glass coverslips. The cover slips containing single attached cells and confluent monolayers were placed in 15 x 45 mm glass tubes and placed on ice for 5 min. Following this incubation at 0°C, the samples were immersed in an alcohol bath at –10°C and allowed to cool for 5 min. Extracellular ice formation was induced at –10°C in the samples using cold forceps. At –10°C, 100% intracellular ice formation has been shown to occur in all of the V-79W and MDCK single attached cells and confluent monolayers (Figure 2.1). The bath was then cooled at 1°C/min to -40°C where the samples were held for 5 min. All samples were then rapidly warmed in a 37°C water bath, incubated in a 5%

alamarBlue solution at 37°C and assessed after 12 or 24 h. Unfrozen samples served as positive growth controls.

Statistical Analysis

Unless otherwise indicated, a one way ANOVA was used with a level of significance set at 0.05.

7.3 RESULTS

Preservation of Metabolic Activity in Single Attached Cells

The metabolic activity of V-79W and MDCK single attached cells following freezing using the three experimental protocols is shown in Figure 7.1. Nucleation at –5°C and cooling at 1°C/min did not result in a high degree of cell recovery. The recovery of metabolic activity following freezing using the standard freezing protocol was 15.0 ± 4.9% for V-79W cells and 5.4 ± 2.4 % for MDCK cells. This is consistent with the results obtained in Chapter 3.

Freezing after equilibration with the cryoprotectant dimethyl sulfoxide results in an increase in the metabolic activity of V-79W and MDCK single attached cells. The recovery following the addition of 10% DMSO was 80.0 ± 10.7 % for V-79W single attached cells and 98.5 ± 3.3% for MDCK single attached cells. This is statistically greater than the results obtained using the standard freezing protocol (V-79W $p<0.001$; MDCK $p<0.001$). The addition and removal of 10% DMSO to the single attached cells in the absence of freezing did not affect cell viability (data not shown).

Nucleating at a lower temperature to produce a higher incidence of intracellular ice resulted in a decrease in the recovery of metabolic activity in both V-79W and MDCK single attached cells. The recovery following nucleation at –10°C and cooling at 1°C/min was 1.1 ± 0.6 % for V-79W and 1.9 ± 1.0 % for MDCK single attached cells. Both values are less than the results from the standard freezing protocol (V-79W $p=0.018$; MDCK $p=0.208$) and the standard freezing protocol with DMSO (V-79W $p<0.001$; MDCK $p<0.001$).

Intracellular Ice Protects Confluent Monolayers

The affect of freezing using the three different experimental protocols for V-79W and MDCK confluent monolayers is shown in Figure 5.2. Using the standard freezing protocol of nucleating at –5 °C and cooling at 1°C/min did not result in a high degree of post-thaw cell recovery. The metabolic activity of confluent monolayers following freezing using the standard protocol was 22.5 ± 9.8% for V-79W and 19.9 ± 10.5 % for MDCK. These results are similar to those obtained for single attached cells (Figure 7.1) and are consistent with the data presented in Chapter 3.

The addition of a cryoprotectant significantly enhanced the recovery of metabolic activity for V-79W and MDCK confluent monolayers. The recovery following the addition of 10% DMSO was 90.5 ± 10.7 % for V-79W single attached cells and 84.6 ± 8.4% for MDCK single attached cells. This is statistically greater than the results obtained using the standard freezing protocol (V-79W $p<0.001$; MDCK $p<0.001$). In the absence of freezing, the addition and removal of 10% DMSO to the confluent monolayers did not affect cell viability (data not shown).

By lowering the nucleation temperature from –5°C to –10°C, formation of intracellular ice was achieved in all of the consitituent cells in the confluent monolayers (Table 4.3 and 4.4). By inducing complete intracellular ice formation, a higher degree of cell recovery could be obtained following slow cooling and rapid thawing. The recovery of metabolic activity was 40.4 ± 3.7 % for V-79W and 58.2 ± 8.4 % for MDCK confluent monolayers. These values are greater than the results obtained using the standard freezing protocol (V-79W $p=0.118$; MDCK $p=0.017$), but much less than the recovery following the addition of DMSO (V-79W $p=0.001$; MDCK $p=0.05$). Intracellular ice in confluent monolayers can provide some degree of protection to the damaging effects of freezing and thawing.

It must be emphasized that the experimental conditions surrounding the formation of intracellular ice in the confluent monolayers were not optimized. No effort was made to maximize the recovery of confluent monolayers that formed intracellular ice by manipulating the experimental design. The recovery of V-79W and MDCK confluent monolayers is dependent on a number of factors including: volume of the surrounding fluid (chapter 3), nucleation temperature, cooling rate, warming rate, time at subzero temperature and physical manipulation. Further studies on the effect these variables have on the recovery of confluent monolayers will enhance the effectiveness of using intracellular ice for cryopreservation.

7.4 DISCUSSION

Inducing intracellular ice is an effective method for the cryopreservation of confluent monolayers. In the absence of any chemical cryoprotectant, a significant degree of cell recovery was obtained following the formation of intracellular ice. While the result was not as great as that obtained using 10% DMSO, it establishes the use of intracellular ice as an effective strategy for the cryopreservation of tissue and tissue model systems.

Intracellular Ice as a Cryoprotectant

By inducing intracellular ice formation in confluent monolayers in the absence of a cryoprotectant, it was possible to obtain a high degree of recovery. As a cryoprotectant can be defined as any agent that affords protection to biological material during freezing and thawing, these results would suggest that intracellular ice is acting as a cryoprotectant. This is a completely novel concept as it directly challenges the current dogma that intracellular ice is lethal.

The mechanism by which intracellular ice functions to protect cells in confluent monolayers is currently unresolved. It has been proposed that the means by which intracellular ice forms in monolayers is critical to post-thaw recovery and not the presence of ice *per se* (4, Chapter 2 and 4). Specifically, the plasma membrane must be maintained for intracellular ice to be innocuous (4, chapter 2 and 4). In Chapter 6 we proposed a model for IIF in confluent monolayers that prevents the disruption of the plasma membrane. The results presented in this study are consistent with that model.

As a 'cryoprotectant', intracellular ice affords significant protection to confluent monolayers during slow cooling. After extracellular ice nucleation and the formation of intracellular ice, the samples were cooled at 1°C/min to –40°C. The fact that there was significantly greater recovery in the samples with intracellular ice supports previous work (chapter 3) demonstrating the effect of intracellular ice in minimizing slow cooling injury. This has important implications in the application of this technique to the cryopreservation of tissues.

The concept of inducing the formation of intracellular ice as a means of affording cryoprotection to tissue model systems is contrary to popular thinking. It has been shown that to minimize damage to cells as a result of IIF, a high subzero nucleation temperature should be chosen (15,37). Working with human oocytes, Trad *et al*. demonstrated superior survival following nucleation of the sample close to the solution melting point (37). We have demonstrated that for confluent monolayers a lower nucleation temperature is preferred as it ensures complete formation of intracellular ice and yields maximum protection. This difference in cryoproservation strategies exemplifies the need for a better understanding of the differences between cells in suspension and the constituent cells of tissues.

Application to Cryopreservation

As tissues are able to tolerate rapid cooling and the formation of intracellular ice, the potential exists to expand current methods of cryopreservation. The current dogma is that slow cooling is the only option available for the cryopreservation of biological material as it limits intra- and extracellular ice formation. Over the past 50 years, this belief has restricted researchers and clinicians from exploring other alternatives for the cryopreservation of cells, tissues and organs. The data presented herein demonstrates that these self-imposed constraints are not necessary.

Rapid cooling of tissues is a valid alternative to the traditional methods for cryopreservation. The results from this study clearly establish the feasibility of utilizing intracellular ice as an alternative to standard slow cooling protocols. This opens up the entire range of temperatures that researchers can now use to develop effective cryopreservation strategies. No longer should researchers be restricted to slow cooling conditions and the avoidance of intracellular ice. In confluent monolayers, and perhaps more complex tissues, rapid freezing and the formation of intracellular ice may be a more effective method of preserving cell and tissue function.

There are a number of advantages to using intracellular ice and rapid freezing in cryopreservation (16). By eliminating the use of chemical cryoprotectants, the complex addition and removal strategies that have been developed are unnecessary. This stands to significantly decrease the pre- and post-processing resources required, permitting the cryopreservation and thawing of more samples in a given time. In addition, removing the chemical additives required in the cryopreservation protocol essentially eliminates problems associated with cryoprotectant toxicity.

As traditional cryopreservation protocols have focused on the rate of cooling to maximize cell survival, these preliminary results would suggest that cooling rate is no longer a concern. Once intracellular ice formation has been induced, the sample may be cooled at whatever rate is feasible in the laboratory or clinical setting. This may include rapid plunge into liquid nitrogen, or simply placing the sample into a –80°C freezer. Warming rates, however, may be much more critical in cells that have formed intracellular ice (16,24, Chapter 3). The utility is that in most laboratories and clinical settings, warming rates are much easier to regulate than controlled-rate cooling. The end result is that the use of intracellular ice in cryopreservation may significantly reduce the equipment and expertise required to freeze and thaw biological material.

While further work remains to optimize the use of intracellular ice in the cryopreservation of tissues and tissue models, the principle has now been established. Induced intracellular ice formation is an effective alternative to classic cryopreservation techniques. As the cryopreservation of tissues and organs has not yet been attained, new ideas or alternatives to current methods of cryopreservation will benefit this developing field. These results provide researchers and clinicians new avenues to explore and new techniques to use in the development of the next generation of cryopreservation protocols.

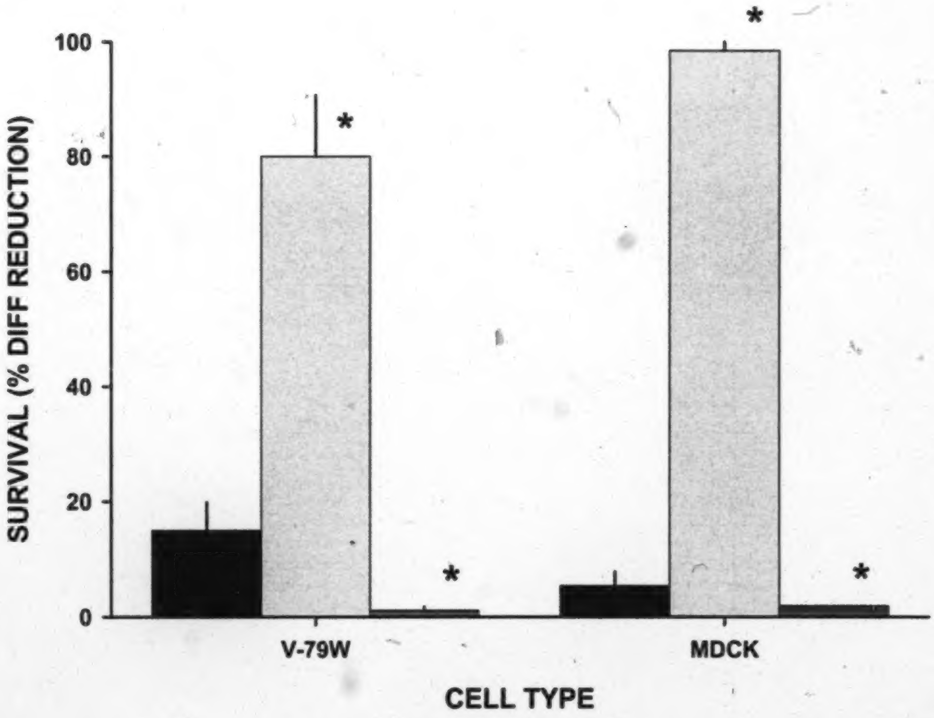

Figure 7.1 – Metabolic activity of V-79W and MDCK **single attached cells** following freezing using three different experimental protocols. The survival of cells that are frozen using the standard freezing protocol (■), the standard freezing protocol + 10% DMSO (▨) or a modified freezing protocol involving intracellular ice (■). The * denotes samples that are significantly different than the standard freezing protocol. Mean ± SEM (n=6).

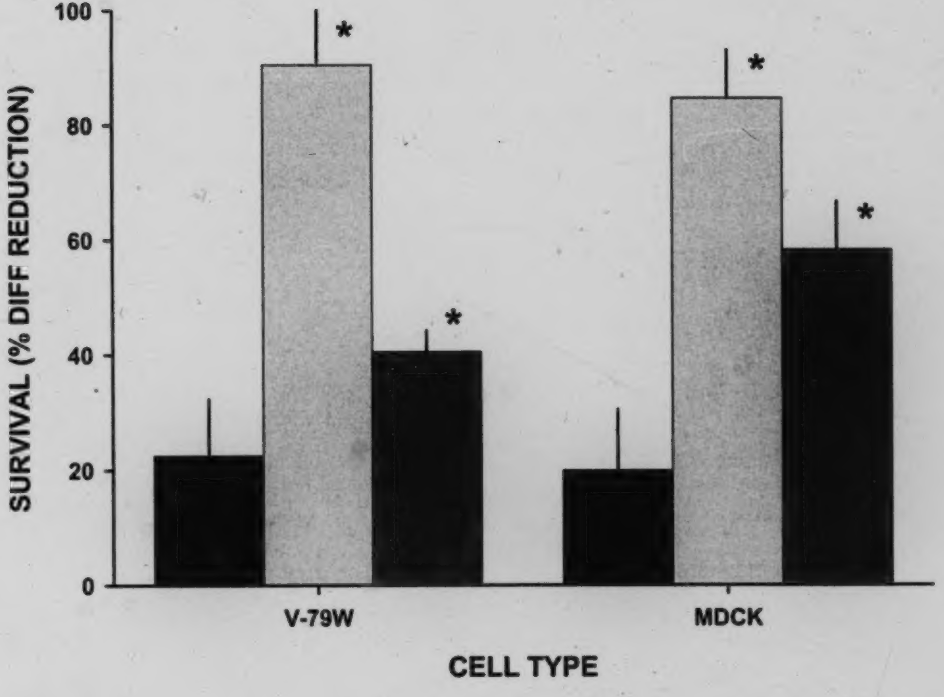

Figure 7.2 – Metabolic activity of V-79W and MDCK **confluent monolayers** following freezing using three different experimental protocols. The survival of monolayers that are frozen using a standard freezing protocol (■), a standard freezing protocol + 10% DMSO (▨) or a modified freezing protocol involving intracellular ice (■). The * denotes samples that are significantly different than the standard freezing protocol. Mean ± SEM (n=6).

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CHAPTER EIGHT

GENERAL DISCUSSION CONCLUSIONS

8.1 SUMMARY OF RESULTS

The work presented in this thesis represents a concerted effort to understand the mechanisms and implications of intracellular ice formation in tissue model systems. Investigations were conducted to explore the nature of the damage that results when ice forms within cells and the effect this intracellular ice has on the response of cells and tissue models to low temperatures. Using a variety of techniques, the mechanism of IIF was examined and correlated with our understanding of the biological response to freezing to produce a conceptual model of ice formation in tissues. The implication of these results was established by examining the role of intracellular ice in the cryopreservation of tissues.

In this thesis we have provided strong evidence to support the conclusion that there are conditions where intracellular ice is innocuous. By employing a novel technique for the progressive assessment of cell viability we were able to dissociate the presence of intracellular ice from lethal cell damage. This allowed us to identify the cell plasma membrane as a critical determinant of lethal injury following intracellular ice formation. In tissue model systems where there is intracellular ice but no membrane damage, then high survival can be achieved.

Expanding our investigation of the innocuous intracellular ice, we examined the effect of IIF on the biological response of the two tissue model systems. In the single attached cells, the formation of intracellular ice resulted in lethal damage and hence the cells were unable to respond favorably to the freezing conditions. However, in confluent monolayers, we demonstrated that under specific conditions intracellular ice could protect cells from the damaging effects of slow cooling.

The first two studies established that the mechanism of formation was critical in determining whether intracellular ice was lethal or innocuous. We followed this up by examining the mechanism by which intracellular ice formation occurs in confluent monolayers to identify the conditions that result in innocuous intracellular ice. Loss of membrane integrity was linked directly to cell death. Using fluorescent cryomicroscopy, we are able to carefully deduce that damage to the plasma membrane occurs during freezing and not during thawing. By performing retrospective analysis on the data, we were also able to correlate the sequence of events involved in the formation and propagation of intracellular ice in confluent monolayers. We determined that the initial formation of intracellular ice results in damage to the plasma membrane. This was then followed by the innocuous, non-random propagation of ice between adjacent cells.

In this thesis we coupled the predictive value of an equation that is analogous to the Kelvin equation with experimental evidence to demonstrate that gap junctions can facilitate the intercellular propagation of intracellular ice. Experimentally we observed a significant change in the low temperature behavior of confluent monolayers at -3°C and suggested that it was the result of the inhibition of gap junction-facilitated ice propagation. This temperature is in the range predicted by theoretical analysis, providing strong evidence to support the pore theory of ice propagation.

We combined all of the information on the conditions surrounding the formation of intracellular ice into a conceptual model. This model represented the first directed effort to incorporate the unique conditions of intracellular ice formation including cell-cell propagation and membrane rupture into a descriptive model for the formation of intracellular ice in two-dimensional tissue systems.

Finally, the importance of the observations and conclusions made in this thesis were highlighted by the use of intracellular ice as a cryoprotectant. Inducing intracellular ice was shown to be an effective method for the cryopreservation of confluent monolayers. In the absence of any chemical cryoprotectant, a significant degree of cell recovery was obtained following the formation of intracellular ice. These results established the use of intracellular ice as an effective strategy for the cryopreservation of tissue and tissue model systems.

8.2 SIGNIFICANCE TO CRYOBIOLOGY

This thesis has made a number of unique contributions that will significantly impact the future of cryobiology.

Using novel techniques to examine the role of the cell plasma membrane and the effect of cell-cell junctions on the formation of intracellular ice, we have demonstrated the ability to study intracellular freezing at a level not previously possible. This work reinforces that the freezing response of a cell in a confluent monolayer is much more complex than the response of cells in suspensions or single attached cells. This has provided much needed information on the unique conditions surrounding the formation of intracellular ice in tissue systems.

This thesis has provided a clearer understanding of the mechanisms responsible for the induction of ice between adjacent cells in a tissue. By formulating the observations on the mechanism of IIF in two-dimensional tissue models into a conceptual framework we have provided the foundation for the development of more effective mathematical models of tissue freezing. With this information, more accurate models can now be developed which may be used to test the ideas presented herein and assist in the development of techniques for the cryopreservation of tissues.

In the process we have presented experimental evidence which has challenged some of the long-standing ideas in cryobiology on the formation of intracellular ice. We have established that intracellular ice can be innocuous and that it is the mechanism by which intracellular ice forms that determines the degree of cell injury. The data presented in this thesis has refined the current theories on the mechanisms of low temperature injury.

We have demonstrated that tissues can tolerate rapid cooling and the formation of intracellular ice, setting up the expansion of current methods of cryopreservation. The results from this thesis clearly establish the feasibility of utilizing intracellular ice as an alternative to standard cryopreservation protocols. This is in direct opposition with the current practice of preventing intracellular ice formation using cryoprotectants and slow cooling. With a better understanding of the protective mechanism of intracellular ice, development of techniques and procedures to minimize cryoinjury can be explored. These results provide researchers and clinicians new avenues to explore and new techniques to use in the development of the next generation of cryopreservation protocols.

APPENDIX A
PROGRESSIVE ASSESSMENT OF CELL VIABILITY FOLLOWING FREEZING[1]

A.1 INTRODUCTION

Effective cryopreservation can significantly enhance the post-thaw recovery of biological function after long-term storage at subzero temperatures. Preserving cells and tissues has been central to the advancement of reproductive biology (12), clinical transplantation (16), and genome resource banking (10,31). However, the freezing and thawing process involved in cryopreservation can severely damage living cells and tissues. Understanding the biological response to low temperatures and identifying the sites of freezing injury are critical to the development of techniques and procedures for the low temperature preservation of living systems.

Damage to cells and tissues during freezing (cryoinjury) is complex and multi-faceted. For individual cells in suspension, numerous sites of freezing injury have been proposed (21) including the plasma membrane, mitochondria, and lysosomes. Each of these cellular components can be damaged by the physical and chemical changes that occur during freezing resulting in lethal injury. In multicellular systems, cell-cell and cell-substrate interactions further complicate the occurrence of cryoinjury (1,2). Assessing the degree of damage resulting from freezing and thawing is therefore obscured by the multiple cellular elements that are affected by the freezing process.

One of the difficult and often controversial topics in low temperature biology is the assessment of cell and tissue "viability" following cryopreservation (3,4,7,9,18,27). The problem is two fold: defining viability, and identifying a means to accurately measure cell viability after exposure to low temperatures. Neither of these factors is easily resolved, and there are numerous factors to consider (3,9). The selection of an appropriate assessment technique for the cells or tissue being investigated typically involves identifying a critical biological function that will be affected by exposure to the experimental conditions. The term "viability" then becomes a function of the single biological process that is being assessed and not necessarily an indicator of the overall health of the cell or tissue. As a result, different assessment techniques will give different measures of cell or tissue 'viability'. Consideration of the cell type and/or tissue function being investigated, the experimental design being conducted and the goal of the investigator are all critical in the choice of viability assessment techniques and the interpretation of experimental data (3).

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As the sites of freeze injury are variable, use of individual assessment techniques may not accurately reflect the state of the cell or tissue following freezing. Single assessment data is typically one-dimensional, providing information specific to the metabolic pathway or cellular structure being examined. In addition, most assessment techniques used result in the irreversible modification or destruction of the biological material being study. This can preclude follow-up measurements (3, 27) or the application of additional tests to determine cell and tissue function. In most cases, long-term proliferation studies can not be conducted on cells pre- and post-freezing using standard assessment techniques. Latent freeze-thaw induced injury or repair of cell injuries is time dependent and will not be detected by assessment techniques that do not consider this temporal factor (20,26). To understand the long-term affects of freezing on multiple biological processes, researchers use numerous independent tests to measure different aspects of cell survival (21). Statistical analysis is then used to draw comparisons and correlations between each of the individual tests in an attempt to understand the overall effects of freezing on the biological system studied. This is generally a cumbersome process requiring complicated experimental designs and lengthy analysis. For these reasons, it has been strongly recommended that the assessment of cryoinjury in cells, tissues and organs be conducted using a concert of assessment techniques that examine multiple biological processes (3,7,18,25,27). Recent efforts are now beginning to explore the use of multi-assessment protocols for the assessment of cell viability (13).

The purpose of this study was to demonstrate the use of multiple, progressive assessment techniques in the determination of cell viability following freezing. This study examined the sequential use of techniques for the assessment of intracellular ice formation, membrane integrity, metabolic function and clonogenic function on freeze-thawed confluent cell monolayers. The effects of freezing and thawing on different biological structures and physiological processes in a single experimental sample were demonstrated using the proposed progressive assessment protocol.

A.2 MATERIALS AND METHODS

Cell Culture

The Madin Darby Canine Kidney (MDCK; CCL34 ATCC) epithelial cell line was used exclusively in this study. The cells were grown at 37°C in an atmosphere of 95% air + 5% carbon dioxide in minimum essential media (MEM) supplemented with 10% v/v fetal bovine serum (all components from GIBCO Laboratories, Grand Island, NY). Cells were kept in tissue culture flasks (25 cm²; Corning Glass Works, Corning, NY) and harvested by exposure to a 0.25% trypsin-EDTA solution (GIBCO) for 10 min at 37°C. The MDCK cells were re-suspended in supplemented MEM to obtain cell suspensions or plated on sterilized cover slips (12 mm circle, Fisher Brand) at a concentration of 2×10^4 cells/mL. The cover slips were kept in petri dishes in an incubator for 3 d to allow the growth of confluent monolayers.

Cryomicroscope and Video System

The cryomicroscope and video system used for this study is described in detail elsewhere (2,23). Briefly, it consisted of a Zeiss fluorescent microscope (Carl Zeiss, Germany), a CCD video camera (ZVS-47DEC, Carl Zeiss), a video recorder (GX4, Panasonic, Japan) and a convection cryostage similar to the design described by Diller *et al.* (6). The cryostage was connected to a computer-controlled interface (Great Canadian Computer Company, Spruce Grove, Canada). The computer monitored the temperature by analyzing the voltage from a thermocouple on the stage and via a proportional controller circuit, heat was added as necessary to allow the stage to follow a user defined thermal protocol.

Assessment Techniques

Intracellular Ice Formation (IIF): If cells or tissues are cooled rapidly, intracellular ice formation will occur. For single cells in suspension, the formation of intracellular ice is an inherently lethal event (15). Avoiding IIF is therefore a critical element in protocols used for the cryopreservation of cells. The formation of intracellular ice results in the scattering of light. The traditional method for the detection of IIF has been the occurrence of a sudden darkening, or 'flashing' within the cytoplasm (22,29). This change in the optical properties of a cell is clearly distinguished using standard brightfield microscopy. An alternative approach has been to fluorometrically assess the formation of intracellular ice (2). Using a fluorescent nucleic acid probe (SYTO 13^{TM}, Molecular Probes, Eugene, OR), intracellular ice formation is evident by a distinct disruption in the pattern of fluorescent staining. Intracellular ice formation was measured using both the 'flash' and fluorometric assessment techniques.

Membrane Integrity: The integrity of the cell plasma membrane has been shown to be critical to the successful survival of cryopreserved cells (19,21,23). Whether damage is caused by, or results from the intracellular freezing process, loss of membrane integrity is an inherently lethal event. A dual fluorescent staining technique was used for the quantitative assessment of the integrity of the cell plasma membrane. SYTO 13, a permeant live cell nucleic acid dye and ethidium bromide (EB; Sigma Chemical Company, Mississuaga, ON) were used to differentially stain the cells. SYTO acts by entering intact cells and labeling both RNA and DNA resulting in a uv excited green emission. EB has been shown to penetrate only cells with damaged membranes and form a fluorescent red complex with nuclear DNA (8). The red EB fluorescence is more pronounced than the green SYTO fluorescence so cells with intact membranes fluoresce green and cells with damaged membranes fluoresce red. The SYTO / EB assay for membrane integrity has been previously shown to correlate well with other assessment techniques for cell viability (1,32). SYTO stains have been used extensively for monitoring the long-term proliferation of living cells and tissues (5,17). Percent survival was calculated as follows:

$$\%\,\text{survival} = \frac{\text{total SYTO positive cells}}{\text{total SYTO positive} + \text{total EB positive cells}} \times 100 \qquad \text{(A-1)}$$

Metabolic Activity: AlamarBlue™ (Biosource International, CA) was used to assess the overall metabolic activity of the confluent monolayers post-thaw. AlamarBlue is a non-toxic oxidation-reduction indicator that changes color in response to the chemical reduction of the growth media as a result of cell metabolism (14,30). This cytotoxicity indictor has been shown to be non-toxic to cells in culture permitting the quantitative measurement of long-term cellular proliferation (24,28,30). AlamarBlue was added to tissue culture media (10% v/v) and MDCK confluent monolayers were incubated in this solution for 12 hours at 37°C. An aliquot of the media (100 µL) was removed and measured on a spectrophotometer (570-600 nm; UVmax, Molecular Dynamics, CA). Cell survival was calculated as the mean percent difference in reduction between the experimental samples and the controls using the following formula (11):

$$\%\,\text{survival} = \frac{A_{LW} - (A_{HW} \times R_o)\,\text{for test well}}{A_{LW} - (A_{HW} \times R_o)\,\text{unfrozen control}} \times 100 \qquad \text{(A-2)}$$

where A_{LW} is the absorbance value of the sample minus the absorbance of the media only at 570 nm, A_{HW} is the absorbance value of the sample minus the absorbance of the media only at 600 nm. R_o given by:

$$R_o = \frac{AO_{LW}}{AO_{HW}} \qquad \text{(A-3)}$$

where AO_{LW} is the absorbance of alamarBlue in media minus the absorbance of media only at 570 and AO_{HW} is the absorbance of alamarBlue in media minus the absorbance media only at 600 nm.

Clonogenic Assessment: The MDCK cells were collected from the coverslips by exposure to a 0.25% trypsin-EDTA solution and diluted to 750 cells/mL.

Tissue culture flasks were seeded with 150 cells/flask and incubated at 37°C for 5 days. The tissue culture media was removed and the colonies were fixed with 70% isopropanol, stained with trypan blue, and rinsed with distilled water before being counted. The formation of cell colonies is an excellent test of the overall ability of a cell population to function and undergo cell division after exposure to low temperatures (20,21). Cell survival was calculated as the mean of the experimental colony counts expressed as a percentage of the mean of controls.

Overall Experimental Design

Confluent cell monolayers were stained with SYTO (1.25 μM) and EB (2.5 μM) and allowed to incubate at 22°C for 10 min. This was previously determined to be sufficient time to allow for the diffusion of the stains (32). The cover slips were placed on the cryostage, and cooled at 25°C/min to –9°C. The sample was then nucleated at the constant temperature using a cold copper probe and held at the experimental temperature for 2 min. The incidence of IIF was determined using the 'flash' method of assessment and the fluorometric assessment. The sample was then warmed at 25°C/min to 22°C and held for 2 min. The integrity of the plasma membrane of each cell in the monolayer was determined using the dual fluorescent staining technique. The entire procedure was videotaped for later analysis. Still images were captured from videotape using the Snappy image capture system (Play Inc., Rancho Cordova, CA) and analyzed using custom designed software.

The samples were then transferred from the cryomicroscope and assayed for metabolic function using the reduction-oxidation indicator alamarBlue. The alamarBlue (10% v/v in supplemented MEM) solution was added to the confluent monolayers and the samples were incubated for 12 h. An aliquot of the solution was removed and measured on a spectrophotometer (570-600 nm). The cells were then trypsinized, plated (150 cells/flask), incubated for 5 days, and the number of colony forming units determined. Throughout the study, unfrozen confluent cell monolayers were used as a positive control group and monolayers placed in boiling water for 1 min served as negative controls. Variations in this experimental procedure will be discussed below.

Statistical Analysis

Throughout the study numerous statistical comparisons were performed. Unless otherwise indicated, a one way ANOVA was used with a level of significance set at 0.05.

A.3 RESULTS

Detection of Intracellular Ice Formation

In order to assess the effectiveness of using fluorescent and brightfield illumination methods to detect intracellular ice formation, the cumulative incidence of IIF as a function of the temperature of nucleation for each detection method was determined. IIF was observed in MDCK cell suspensions and confluent cell monolayers frozen to defined experimental subzero temperatures (Figure A.1; -3 to –11°C). The IIF characteristics of cell suspensions were included to demonstrate that the detection method for IIF was not affected by the morphology of the cells resulting from culture. Cells in suspension displayed a gradual increase in the cumulative incidence of IIF with 100% formation occurring at –13°C. This is in agreement with other studies documenting the formation of intracellular ice in suspensions of various cell types (1,15,23). At all experimental temperatures there was complete formation of IIF in the constituent cells of a confluent monolayer. These results are consistent with studies documenting the IIF behavior of MDCK epithelial cells in culture (1). In both the cell suspensions and confluent cell monolayers there was no statistical difference in the method used to detect intracellular ice formation (p=0.968). The addition of the fluorescent indicator to the cells prior to freezing did not affect the incidence of IIF. A nucleation temperature of –9°C was selected for the remainder of the experiments as this temperature ensures complete formation of IIF in the MDCK confluent monolayers.

Assessment of Membrane Integrity

After assessment of intracellular ice formation, the incidence of membrane damage was determined on the same specimen using the SYTO / EB dual fluorescent staining technique. The time course for membrane-related cryoinjury in the MDCK confluent cell monolayers is shown in Figure A.2. A total of eight samples (300 cells / sample) were cooled to –9°C, frozen and then thawed and incubated at 37°C. Unfrozen and boiled samples served as positive and negative controls, respectively. The number of cells that displayed an intact plasma membrane (score SYTO positive) were determined after 0, 2, 4, 6 and 12 h incubation and percent survival was calculated. Approximately 25% of the cells in the confluent cell monolayer displayed a ruptured cell membrane post thaw. This is consistent with other work describing the affect of cell adhesions on membrane integrity (2). There was no statistical difference in the survival of confluent cell monolayers over the 12 h incubation period ($p=0.963$).

Assessment of Metabolic Activity

In the progressive assessment technique, the determination of membrane integrity is followed by an evaluation of the overall metabolic activity of the constituent cells in the confluent cell monolayers. The time course for the reduction of alamarBlue following incubation at 37°C for the confluent cell monolayers is shown in Figure A.3. AlamarBlue reduction is not initially measurable until after 4 h incubation. Apparent survival reaches a minimum between 8 and 16 h whereafter an increase in the survival is observed. This increase is likely due to the increase in active cell number as a result of normal cell division and/or an increase in cell metabolism as a result of the repair of any sublethal freezing injury. Selecting a 12-hour incubation ensures that the assessment will be conducted within the minimum survival range.

Comparing the results of the membrane integrity assay from Figure A.2 with the percent survival of metabolic activity after 12 h incubation shows no statistical difference ($p=0.547$). Both the membrane integrity procedure and the metabolic activity technique are equally effective at measuring the damage occurring in confluent cell monolayers after intracellular ice formation.

Interaction between Assessment of Membrane Integrity and Metabolic Function

Critical to the validation of the progressive assessment technique is the determination of potential interactions between the various measurement procedures. The first interaction investigated was the effect of fluorescent indicators SYTO and ethidium bromide on the reduction of alamarBlue. Confluent cell monolayers where frozen in the presence or absence of SYTO / EB, incubated in alamarBlue, and measured after 4 h (Table A.1). There was no statistical difference in the survival measures (p=0.230), indicating that the presence of SYTO / EB did not affect the results of the alamarBlue assessment.

The second interaction studied was the effect of alamarBlue on membrane integrity. This reversal experiment involved assessing the membrane integrity of confluent cell monolayers after a 4-hour incubation with or without alamarBlue. Table A.2 shows that assessment of membrane integrity is not affected by incubation with alamarBlue (p=0.910). This further demonstrates that there was no interference of the different chemical indicators.

Assessment of Clonogenic Function

The final assessment technique performed as part of the progressive assessment procedure was the determination of clonogenic function. After treatment and incubation in alamarBlue, the confluent cell monolayers were trypsinized, plated for colony growth and incubated for 5 days. Over 80% survival (Figure A.4; +SYTO/EB, +AB) was obtained following this procedure indicating that the constituent cells of the confluent cell monolayers frozen at –9°C were able to divide and form colonies post-thaw. The results of the clonogenic assay agree with those determined using the membrane integrity test (p=0.617) and the post-thaw measurement of metabolic activity (p=0.704).

The effect of the chemical reagents used to determine membrane integrity and metabolic function on clonogenic activity was determined (Figure A.4). In the absence of both SYTO / EB and alamarBlue, there was no difference in survival compared to the results from the complete progressive assessment procedure (p=0.971). There was also no significant difference in the results when either the membrane integrity (p=0.714) or the metabolic activity assessments (p=0.700) were excluded. Together, this evidence demonstrates that the progressive use of SYTO / EB and alamarBlue does not affect the results obtained using the clonogenic assay.

A.4 DISCUSSION

This study has demonstrated the use of a novel experimental procedure for the progressive assessment of cryoinjury in attached confluent cell monolayers following freezing. Through the combined use of multiple techniques on a single experimental specimen, the effects of exposure to low temperatures on a number of specific biological structures and processes can be determined. This experimental technique allows investigation of the effects of freezing on the incidence of intracellular ice formation, the integrity of the cell plasma membrane, the post-thaw metabolic activity of the cells and the ability for cells to divide and form colonies. Together this information allows for detailed information on different sites of freezing damage to be studied.

Assessment techniques were selected to minimize or eliminate interactions, and reagents were selected from those used for long-term proliferation studies. From the data, it is clear that the use of the membrane integrity indicators SYTO / EB, the metabolic activity reagent alamarBlue, and the clonogenic assay did not interact or negatively effect the ability of each to resolve cell survival following intracellular ice formation.

Progressive assessment is an important advancement in the measurement of cell viability following experimental manipulation. Combining these multiple assessments into a single procedure results in a more accurate reflection of the "viability" of the cell or tissue. Further, the application of this progressive assessment eliminates experimental work as a multitude of viability data can be gathered on a single specimen reducing the number of samples required. By incorporating a number of different assays into a single experimental procedure, limitations and errors associated with one technique can be offset by the use of another. This is a powerful tool that will significantly contribute to developing an understanding of the low temperature response of biological systems.

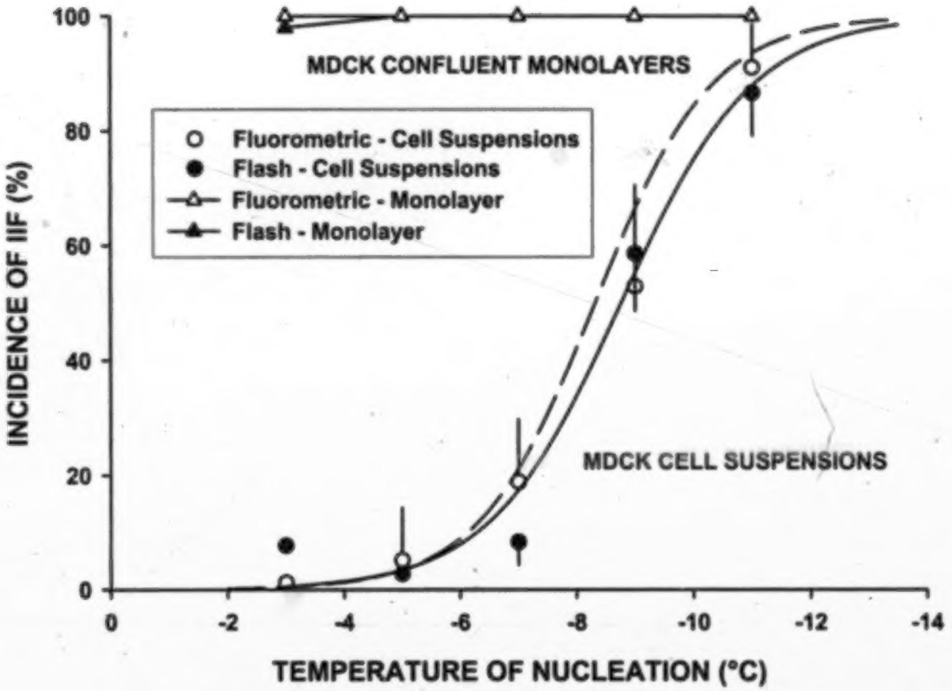

Figure A.1 – Detection of intracellular ice formation in MDCK confluent monolayers and cell suspensions. The cumulative incidence of IIF as a function of temperature of nucleation for the standard 'flash' method of detection and the fluorometric technique using SYTO 13 is shown. Mean ± SEM (n=3).

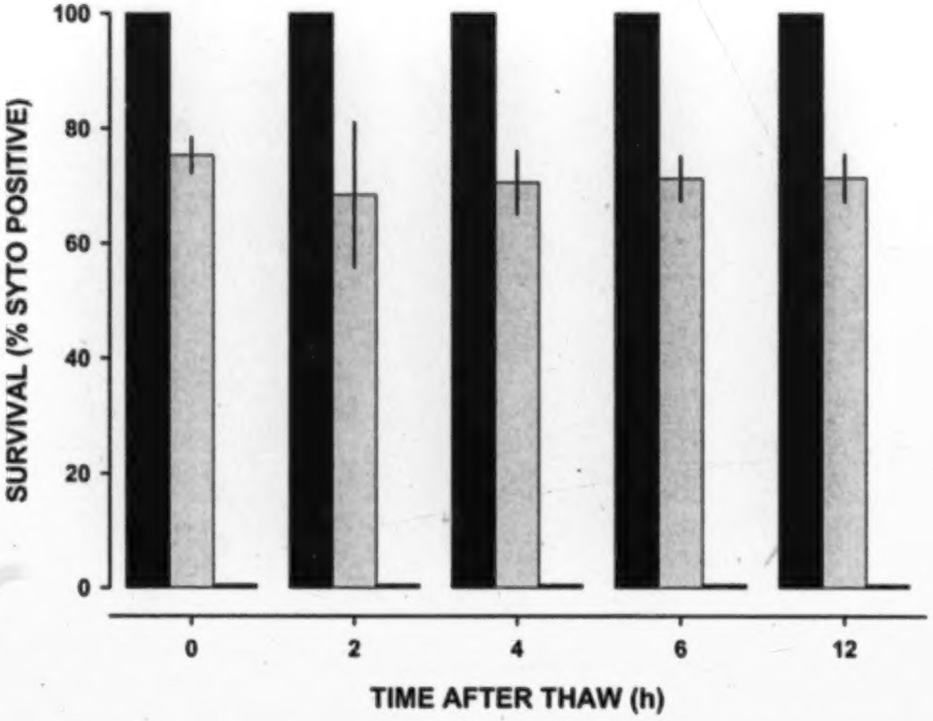

Figure A.2 – Time course for membrane-related cryoinjury in MDCK confluent monolayers assessed using SYTO/EB. The survival of confluent monolayers that are unfrozen (■), nucleated and frozen at -9°C (▨) or boiled (■ is plotted as a function of time after thaw. Mean ± SEM (n=8).

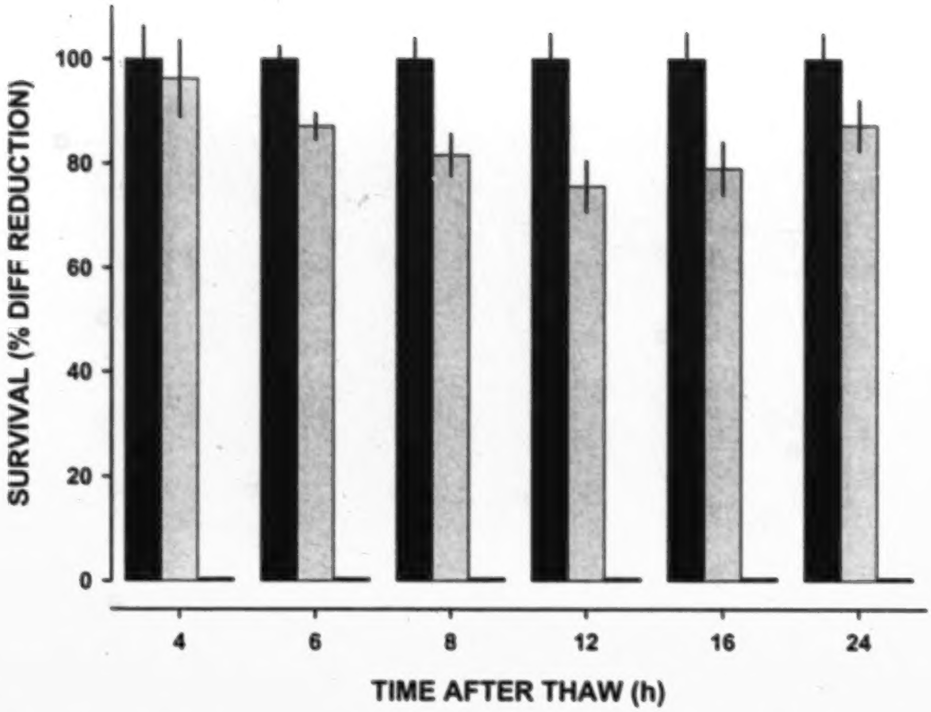

Figure A.3 – Time course for the reduction of alamarBlue by MDCK confluent monolayers following post-thaw incubation. Survival of confluent monolayers that were unfrozen (■), nucleated and frozen at -9°C (▨) or boiled (■ is plotted as a function of time after thaw. Mean ± SEM (n=8).

	Survival (% Diff. Reduction) Mean ± SEM (n=6)	
	with SYTO / EB	**without SYTO / EB**
unfrozen	100.0 ± 3.2	100.0 ± 5.6
-9 °C	91.8 ± 3.8	92.9 ± 9.6
boiled	0.0 ± 0.5	0.0 ± 0.7

Table A.1 – Effect of SYTO / EB on alamarBlue reduction. MDCK confluent monolayers were frozen in the presence (with SYTO/ EB) or absence (without SYTO / EB) of the fluorescent indicators SYTO 13 and ethidium bromide and then assessed using alamarBlue.

	Survival (% SYTO Positive) Mean ± SEM (n=6)	
	SYTO / EB pre AB	**SYTO / EB post AB**
unfrozen	100.0 ± 0.0	100.0 ± 0.0
-9 °C	75.4 ± 3.1	81.0 ± 1.5
boiled	0.0 ± 0.0	0.0 ± 0.0

Table A.2 – Effect of alamarBlue on the assessment of membrane integrity. The assessment of membrane damage was conducted before (SYTO / EB pre AB) or after (SYTO/EB post AB) the addition of alamarBlue.

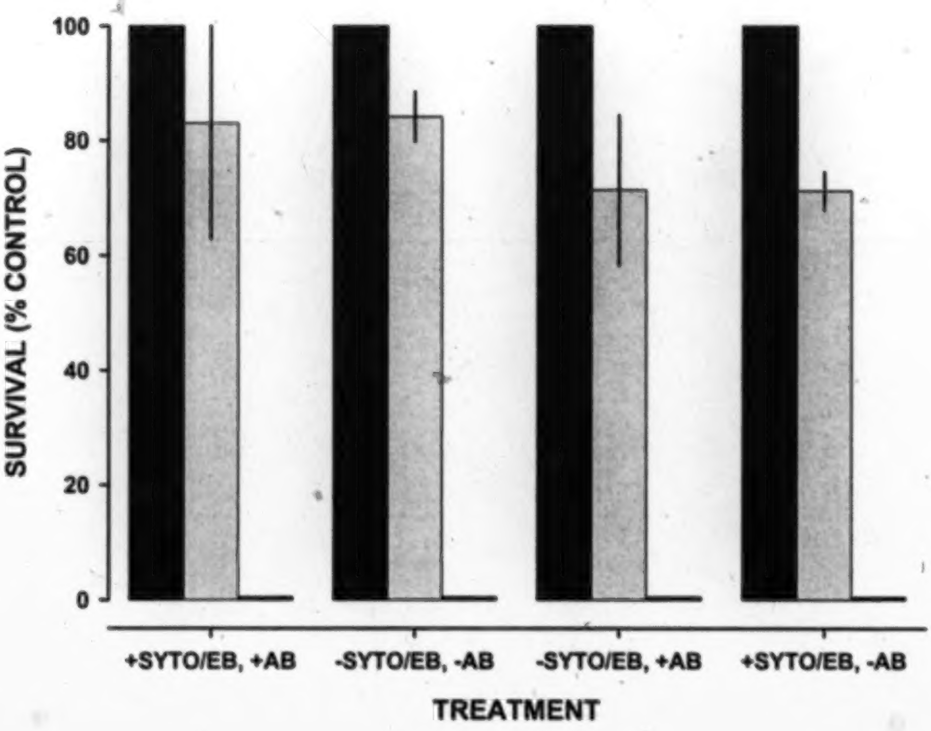

Figure A.4 – Clonogenic function of MDCK monolayers following nucleation at -9°C. Survival of cells following the complete progressive assessment technique (+SYTO / EB, +AB) is compared to the results obtained when the membrane integrity and metabolic activity tests are excluded (-SYTO / EB, - AB) and the results when just membrane integrity (-SYTO / EB, +AB) or just metabolic activity (+SYTO / EB, - AB) are excluded. Survival of confluent monolayers that were unfrozen (■), nucleated and frozen at -9°C (▨) or boiled (■) is shown. Mean ± SEM (n=6).

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APPENDIX B
PHYSICAL PARAMETERS

These physical parameters were used in Chapter 5.

Constant	Symbol	Value
freezing point (at 1 atm)	T^{o}_{fp}	273.15 K
interfacial tension	σ_{SL}	1.7 to 2.0 x 10^{-2} J/m^2
molar volume of ice	v_2	1.97 x 10^{-5} m^3/mol
molar volume of liquid water	v_3	1.80 x 10^{-5} m^3/mol
molar heat of fusion	L_f	-22.0 J/mol K